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Award Number: DAMD17-99-1-9027

TITLE: Investigation of the Candidate Tumor Suppressor Gene prk in Prostate Cancer

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REPORT DATE: December 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2001	Annual (1 Nov 00 - 31 Oct 01)

4. TITLE AND SUBTITLE
Investigation of the Candidate Tumor Suppressor Gene prk in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9027

6. AUTHOR(S)
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American Health Foundation
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

14. SUBJECT TERMS
Prostate Cancer

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

Prostate cancer is the most common malignancy among American men. Yet our understanding of the genetic events which occur during the course of the development of this disease has lagged behind that for other common tumors. Recent studies suggest that prostate cancer appears to be driven by the mutation of as yet unknown genes. The identification of such genes should be essential to the understanding of the biology and pathology of this disease. Allelotype analysis of prostate cancer has implicated several loci of candidate tumor suppressor genes (1-3). For example, loss of heterozygosity of polymorphic markers has been identified at 7q, 8p, 10, 16q and 18q (2,3). Of these, 8p is among the most frequently lost in prostate carcinoma (1-3). Detailed analyses have defined the region of allele loss in prostate and other cancers to 8p12-21 and 8p22-pter (1,4), suggesting that the presence of two or more tumor suppressor genes at this genomic locus.

We have identified a gene, *Plk3* (previously termed *Prk*), encoding a protein serine/threonine kinase (5-7). Structural analysis has revealed that *Plk3* is homologous to the budding yeast Cdc5 and *Drosophila melanoganster* polo, both of which have been implicated in regulating G_2/M transition and M phase progression. Mutations of Cdc5 in the budding yeast render the yeast unable to complete mitosis at the restrictive temperature. However, this temperature sensitive phenotype can be rescued by the ectopic expression of human *Plk3* gene (6). Northern blotting analysis of samples from eighteen lung cancer patients has revealed that *Plk3* mRNA is significantly down-regulated in tumor tissues as compared with those of uninvolved tissues (5). FISH analysis has mapped the *Plk3* gene to 8p21 (8).

The observations that *Plk3* has a function during the cell cycle, similar to known tumor suppressor genes such as RB, p53, and p16, and that it is localized at a site frequently deleted in prostate carcinomas prompted us to test whether *Plk3* may be a novel tumor suppressor gene.

RESULTS (Body)

I. Plk3 is involved in DNA damage checkpoint response pathway (9)

We have shows that upon hydrogen peroxide (H_2O_2) treatment phosphorylation of p53 was rapidly induced in GM00637D cells and this phosphorylation occurred on serine-9, serine-15, serine-20, but not on serine-392. In addition, H_2O_2-induced phopshorylation of p53 was followed by induction of p21, suggesting functional activation of p53. Induction of phosphorylation of p53 on multiple serine residues by H_2O_2 was caffeine-sensitive and blocked in $ATM^{-/-}$ cells. Polo-like kinase-3 (Plk3) activity was also activated upon H_2O_2 treatment and this activation was ATM-dependent. Recombinant His_6-Plk3 phosphorylated glutathione-*S*-transferase (GST)-p53 fusion protein but not GST alone. When phoshorylated in vitro by His_6-Plk3, but not by the kinase defective-mutant His6-$Plk3^{K52R}$, GST-p53 was recognized by an antibody specifically to serine-20 phosphorylated p53, indicating that serine-20 is an in vitro target of Plk3. Also serine-20-phosphorylated p53 was coimmunoprecipitated with Plk3 in cells treated with H_2O_2. Furthermore, although H_2O_2 strongly induced serine-15 phosphorylation of p53, it failed to induce serine-20 phosphorylation in Plk3-dificient Daudi cells. Ectopic expression of a Plk3 dominant negative mutant, $Plk3^{K52R}$, in GM00637D cells suppressed H_2O_2-induced serine-20 phosphorylation. Taken together, our studies strongly suggest that the oxidative stress-induced activation of p53 is at least in part mediated by Plk3.

II. Plk3 directly phosphorylates and regulates p53 (10)

Polo-like kinase 3 contributes to regulation of M phase of the cell cycle (1). Plk3 physically interacts with Cdc25C and phosphorylates this protein phosphatase predominantly on serine-216 (2), suggesting that the role of Plk3 in mitosis is mediated, at least in part, through direct regulation of Cdc25C. We have demonstrated that ectopic expression of a kinase active Plk3 (Plk3-A) induced apoptosis. In response to DNA damage, the kinase activity of Plk3 was rapidly increased in an ATM-dependent manner whereas that of Plk1 was markedly inhibited. Recombinant Plk3 phosphorylated in vitro a glutathione *S*-transferase (GST) fusion protein containing p53, but not GST alone. Recombinant Plk1 also phosphorylated p53 but on residues that differed from those targeted by Plk3. Co-immunoprecipitation and pull-down assays demonstrated that Plk3 physically interacted with p53 and that this interaction was enhanced upon DNA damage. In vitro kinase assays followed by immunoblotting showed that serine-20 of p53 was a target of Plk3. Furthermore, expression of a kinase-defective Plk3 mutant ($Plk3^{K52R}$) resulted in significant reduction of p53 phosphorylation on serine-20, which was correlated with a decrease in the expression of p21 and with a concomitant increase in cell proliferation. These results strongly suggest that Plk3 functionally links DNA damage to cell cycle arrest and apoptosis via the p53 pathway.

KEY RESEARCH ACCOMPLISHMENTS

- Plk3 kinase activity is rapidly activated upon genotoxic stress.
- Genotoxic stress-activated Plk3 is ATM-dependent.
- Plk3 phosphorylates p53 on serine 20 in vitro.
- Plk3 may regulate p53 phosphorylation on serine-20 in vivo.

REPORTABLE OUTCOMES

1. S-Q Xie, Q Wang, H-Y Wu, Lu L, M. Jhanwar-Uniya,l **W Dai**. Reactive oxygen species induces phosphorylation of p53 on serine-20 is mediated at least in part by Plk3. 2001 *J. Biol. Chem.* 276:36194-36201.
2. H-Y Wu, S-Q Xie, Q Wang, J Cogswell, C Conn, PJ Stambrook, **W Dai**. p53 as a target of Plk3 during activation of the DNA damage checkpoint. *J. Biol. Chem.* 2001 276:43305-12.
3. S-Q Xie, Q Wang, L Lu, **W Dai**. Proteasome-dependent down-regulation of $p21^{Waf1/Cip1}$ induced by reactive oxygen species. *Exp. Cell Res.* 2001 (under review)

CONCLUSIONS

Growth suppression induced by Plk3 is partly mediated through the p53 pathway.

APPENDICES

A. Reprints/preprint

1. S-Q Xie, Q Wang, H-Y Wu, Lu L, M. Jhanwar-Uniya,l **W Dai**. Reactive oxygen species induces phosphorylation of p53 on serine-20 is mediated at least in part by Plk3. 2001 *J. Biol. Chem.* 276:36194-36201.
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9. H-Y Wu, S-Q Xie, Q Wang, J Cogswell, C Conn, PJ Stambrook, **W Dai**. p53 as a target of Plk3 during activation of the DNA damage checkpoint. *J. Biol. Chem.* 2001 276:43305-12.
10. S-Q Xie, Q Wang, H-Y Wu, Lu L, M. Jhanwar-Uniya,l W Dai. Reactive oxygen species induces phosphorylation of p53 on serine-20 is mediated at least in part by Plk3. 2001 *J. Biol. Chem.* 276:36194-36201.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 39, Issue of September 28, pp. 36194–36199, 2001

Printed in U.S.A.

Reactive Oxygen Species-induced Phosphorylation of p53 on Serine 20 Is Mediated in Part by Polo-like Kinase-3*

Received for publication, May 8, 2001, and in revised form, July 3, 2001
Published, JBC Papers in Press, July 10, 2001, DOI 10.1074/jbc.M104157200

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Upon exposure of cells to hydrogen peroxide (H_2O_2) phosphorylation of p53 was rapidly induced in human fibroblast GM00637, and this phosphorylation occurred on serine 9, serine 15, serine 20, but not on serine 392. In addition, H_2O_2-induced phosphorylation of p53 was followed by induction of p21, suggesting functional activation of p53. Induction of phosphorylation of p53 on multiple serine residues by H_2O_2 was caffeine-sensitive and blocked in $ATM^{-/-}$ cells. Polo-like kinase-3 (Plk3) activity was also activated upon H_2O_2 treatment, and this activation was ATM-dependent. Recombinant His_6-Plk3 phosphorylated glutathione *S*-transferase (GST)-p53 fusion protein but not GST alone. When phoshorylated *in vitro* by His_6-Plk3, but not by the kinase-defective mutant $His6\text{-}Plk3^{K52R}$, GST-p53 was recognized by an antibody specifically to serine 20-phosphorylated p53, indicating that serine 20 is an *in vitro* target of Plk3. Also serine 20-phosphorylated p53 was coimmunoprecipitated with Plk3 in cells treated with H_2O_2. Furthermore, although H_2O_2 strongly induced serine 15 phosphorylation of p53, it failed to induce serine 20 phosphorylation in Plk3-dificient Daudi cells. Ectopic expression of a Plk3 dominant negative mutant, $Plk3^{K52R}$, in GM00637 cells suppressed H_2O_2-induced serine 20 phosphorylation. Taken together, our studies strongly suggest that the oxidative stress-induced activation of p53 is at least in part mediated by Plk3.

Reactive oxygen species (ROS),[1] ubiquitously present, are very reactive and cause damage to biological molecules, including DNA. ROS are potentially mutagenic and may be involved in activation of protooncogene and inactivation of tumor suppressor genes (1, 2). Thus, ROS are suspected to represent important human carcinogens (3, 4). Oxidative signals, either external or internal, are thought to be detected by sensor molecules and mediated by cellular signal transduction systems, which eventually results in cell cycle arrest, senescence, or apoptosis in normal diploid fibroblast cells. ATM has been proposed to be a sensor of oxidative damage of cellular macromolecules such as DNA (5). The tumor suppressor protein p53 appears to be a major effector of the genotoxic stress-signaling pathway that is mediated by ATM (6). In fibroblast cells, p53 protein level is increased upon H_2O_2 treatment, and the level of p53 is correlated with replicative senescence and apoptosis (7). In the $p66shc^{-/-}$ cells, p53 activation and its target gene p21 expression are impaired in response to oxidative stress (8). However, a p53-independent pathway that mediates H_2O_2-induced G_2/M growth arrest has also been reported (9).

Members of the Polo family of protein kinases, conserved through evolution, have been characterized in yeast (10), *Caenorhabditis elegans* (11), *Drosophila melanogaster* (12), *Xenopus laevis* (13), mouse (14, 15), and human (16, 17). The founding member of this family, Polo, was originally identified in the fruit fly and was shown to be a serine-threonine kinase required for mitosis (12). Mammalian cells contain at least three proteins (Plk1, Plk2, and Plk3) that exhibit marked sequence homology to Polo (14, 15, 18, 19). As cells progress through the cell cycle, Plk proteins undergo substantial changes in abundance, kinase activity, or subcellular localization. In human cells, the amounts of Plk1 protein and its kinase activity peak at mitosis (18). During mitosis, Plk1 transiently associates with mitotic structures such as the spindle apparatus, kinetochores, and centrosomes (20). Recent studies have shown that Plk1 contributes to a variety of mitotic (or meiotic) events, including activation of cyclin B-Cdc2, breakdown of the nuclear membrane, centrosome maturation, and formation of the bipolar spindle at the onset of mitosis (21–23). Plk1 also controls the exit of cells from mitosis by regulation of the anaphase-promoting complex (24). Plk3 shows little resemblance to Plk1 with regard to function in mammalian cell cycle regulation. Thus, the abundance of Plk3 remains relatively constant during the cell cycle, and its kinase activity peaks during late S and G_2 phases (25). Furthermore, Plk3 phosphorylates Cdc25C on serine 216, resulting in inhibition of the activity of this protein (25), whereas phosphorylation of Cdc25C by Plx1, a *Xenopus* Plk1 ortholog, results in activation of this protein (13).

Polo family kinases also participate in the response to DNA damage (26–28). For example, Cdc5, a budding yeast ortholog of *Drosophila* polo, promotes adaptation to cell cycle arrest at the DNA damage checkpoint (29). The electrophoretic mobility of Cdc5 in denaturing gels is affected by prior subjection of cells to DNA damage, and this modification is dependent on Mec1, Rad53 (a yeast Chk1 homolog), and Rad9 (26). In addition, a functionally defective Cdc5 mutant protein suppresses a Rad53 checkpoint defect, whereas overexpression of Cdc5 overrides checkpoint-induced cell cycle arrest (27), suggesting that Cdc5 acts downstream of Rad53. Moreover, DNA damage appears to interfere with the activation of Plk1 in mammalian cells, resulting in down-regulation of the kinase activity of this protein. In contrast, expression of dominant negative mutants of Plk1

* This work was supported in part by Public Health Service Award CA47729 (to W. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ROS, reactive oxygen species; IR, ionizing radiation.

overrides the induction of G_2 arrest by DNA damage (28).

We have been studying the biological role of polo-like kinase-3 (Plk3, previously named Prk) during normal and abnormal cell growth (17, 25, 30, 31). Here we report that ROS induces activation of Plk3 as well as p53, which is correlated with p53 phosphorylation on multiple serine sites. Activation of both Plk3 and p53 is ATM-dependent. In addition, we have obtained experimental evidence strongly suggesting that Plk3 mediates ROS-induced serine 20 phosphorylation of p53.

MATERIALS AND METHODS

Cell Culture—Various cell lines, including ATM-deficient cell line (ATCC number CRL-1702), were purchased from ATCC. CRL-1702 has been characterized as $ATM^{-/-}$ (32). GM00637 cell line (human fibroblast) was originally from the Coriell Institute for Medical Research. HeLa, A549, GM00637, DU145, LNCap, and PC-3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml penicillin and 50 μg/ml streptomycin sulfate) with 5% CO_2. DAMI, HEL, and HL-60 cells were cultured in RPMI 1640 medium, and Daudi cells were culture in McCoy's medium supplemented with fetal bovine serum and antibiotics as above.

Immunoblotting—Cells treated with H_2O_2 (200 μM unless otherwise specified) or adriamycin (100 μM) were collected and lysed (25). In some experiments, caffeine (2 μM) was supplemented to the cultured cells for 30 min prior to the treatment with H_2O_2 or adriamycin. Equal amounts (40 μg) of protein lysates from the treated cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies (New England Biolabs) to phosphorylated p53 (specifically phosphorylated on serine 9, serine 15, or serine 20), p21, or Bax. The same blots were also stripped and reprobed with antibodies to regular p53 (Santa Cruz Biotechnology). Signals were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Protein Kinase Assays—Immunocomplex kinase assays were performed essentially as described previously (25). In brief, A549 cells were exposed to H_2O_2 (200 μM) for various times, lysed, and subjected to immunoprecipitation with antibodies to Plk3. The resulting precipitates were resuspended in a kinase buffer (10 mM Hepes-NaOH (pH 7.4), 10 μM $MnCl_2$, 5 mM $MgCl_2$), and the kinase reaction was initiated by the addition of [γ-^{32}P]ATP (2 μCi) (Amersham Pharmacia Biotech) and α-casein (Sigma). After incubation for 30 min at 37 °C, the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Recombinant His_6-Plk3, produced and purified as described previously (25, 30), was assayed for kinase activity as a positive control. In some kinase assays, GST-p53 was incubated with His_6-Plk3 or His_6-Plk3^{K52R} in the kinase buffer supplemented with "cold" ATP. After reaction, Plk3-phosphorylated GST-p53 samples, as well as nonphosphorylated GST-Plk3, were blotted for serine 20 phosphorylation.

Coimmunoprecipitation Analysis—GM00637 cell lysates were incubated for 30 min at room temperature in a total volume of 500 μl with of 20 μl of protein A/G-agarose bead slurry (Santa Cruz Biotechnology). After removal of the beads, the supernatant was supplemented with either rabbit polyclonal (PharMingen) or mouse monoclonal antibodies to Plk3, or with control immunoglobulins followed by incubation with constant agitation for overnight at 4 °C. Protein A/G-agarose beads (20 μl) were then added to each immunoprecipitation mixture, and the incubation was continued for 1 h at room temperature. Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to immunoblot analysis with monoclonal antibodies to serine 20-phosphorylated p53.

Transient Transfection—GM00637 cells were transfected, using the LipofectAMINE method (Life Technologies, Inc.), with constructs expressing Plk3 or Plk3^{K52R25} or with the vector pCR592. One day after transfection, cells were treated with or without H_2O_2 for 30 min. Cell lysates were prepared and blotted for Plk3, p53, or serine 20-phosphorylated p53.

RESULTS

Although recent studies have shown that phosphorylation of p53 plays an important role in stabilization and activation of this tumor suppressor protein in cells exposed to ionizing radiation (IR) or UV (6, 33), the mechanism by which ROS-induced p53 accumulation/activation remains unclear (34). To determine whether ROS activates p53 through phosphorylation, GM00637D cells were exposed to H_2O_2 for various times, and p53 phosphorylation status was analyzed by immunoblotting using phospho-specific antibodies. Fig. 1*A* shows that upon H_2O_2 treatment, p53 was rapidly phosphorylated on serine 20 and serine 15 in GM00637D cells. Serine 9 phosphorylation was also induced with a slow kinetics (Fig. 1*A*, *lane 5*). However, serine 392 phosphorylation was not detected (data not shown). These results indicate that p53 accumulation upon oxidative stress as reported by von Harsdorf and Dietz (35) is at least partly due to phosphorylation of p53 on serine 15 and serine 20, because these two residues are located within the domain of the protein that interacts with HDM2 (human ortholog of murine double minute-2 protein, MDM2), resulting in stabilization of the normally short-lived p53 protein in response to the stress (36).

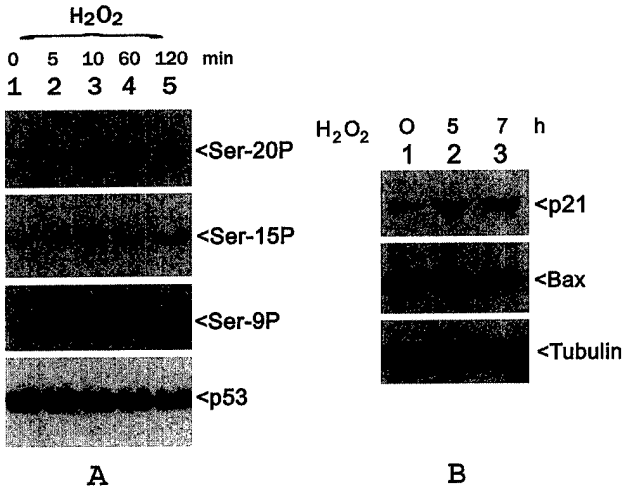

FIG. 1. **H_2O_2 induces phosphorylation and activation of p53.** *A*, GM00637 cells treated with H_2O_2 for the indicated times were collected, and equal amounts of cell lysates were analyzed for p53 phosphorylation via Western blotting using antibodies specific to phosphorylated serine 9, serine 15, or serine 20. The same blot was stripped and reprobed with antibody to all forms of p53. *B*, GM00637D cells were treated with H_2O_2 for the indicated time, and equal amounts of cell lysates were blotted for p21, Bax, or α-tubulin.

Phosphorylation and activation of p53 upon challenge with genotoxic stress such as IR and UV often results in cell cycle arrest (6). In fact, the trans-activation by p53 of genes such as those encoding p21 and Bax proteins is thought to be responsible at least in part for cell cycle arrest and apoptosis, respectively, in cells subjected to genotoxic stress (37). To determine whether H_2O_2-induced p53 phosphorylation is correlated with its functional activation, we measured expression of its target genes p21 and Bax. Fig. 1*B* shows that 5 h after H_2O_2 treatment, the p21 protein level began to increase (*lane 2*) and that 7 h post-treatment it was more than quadrupled (*lane 3*) compared with the untreated control (*lane 1*). On the other hand, little increase in Bax protein levels were observed in cells treated with H_2O_2.

DNA damage caused by IR activates p53 through phosphorylation on multiple residues, and this activation is ATM-dependent (33). To determine whether ROS-induced p53 phosphorylation was also ATM-dependent, we treated GM00637 cells with caffeine, an ATM/ATR inhibitor, prior to exposure of the cells to H_2O_2 or IR-mimetic drug adriamycin. Fig. 2*A* shows that caffeine (*CFN*) partially blocked H_2O_2-stimulated phosphorylation of p53 on serine 15 and serine 20 (*lanes 2* and *5*), whereas it completely inhibited adriamycin (*ADR*)-induced phosphorylation of p53 on all three residues (*lanes 3* and *6*).

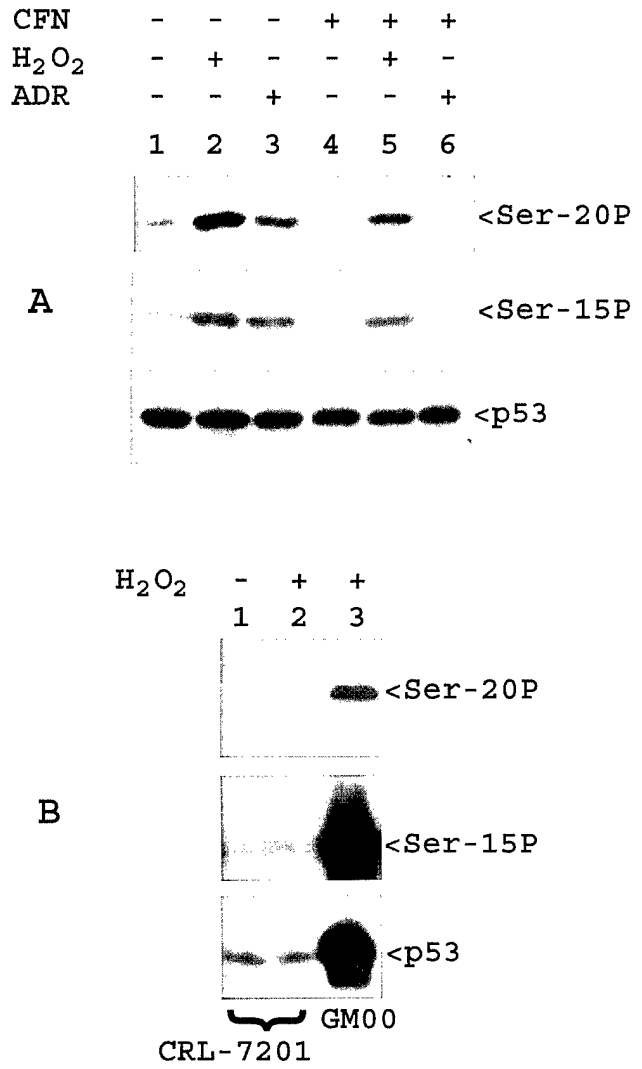

FIG. 2. **H_2O_2-induced p53 phosphorylation is ATM-dependent.** *A*, GM00637 cells pretreated with caffeine (*CFN*) were exposed to H_2O_2 or adriamycin (*ADR*) for 30 min. Equal amounts of protein lysates from the treated cells were analyzed for p53 phosphorylation using phospho-specific antibodies to serine 15 or serine 20. The same cell lysates were also blotted for all forms of p53. *B*, ATM-deficient cells (*CRL-1702*) were treated with H_2O_2 for 30 min, and equal amounts of cell lysates were blotted with antibodies to serine 20-phosphorylated or serine 15-phosphorylated p53, as well as all forms of p53. GM00637 cell lysates (*lane 3*) were used as positive control.

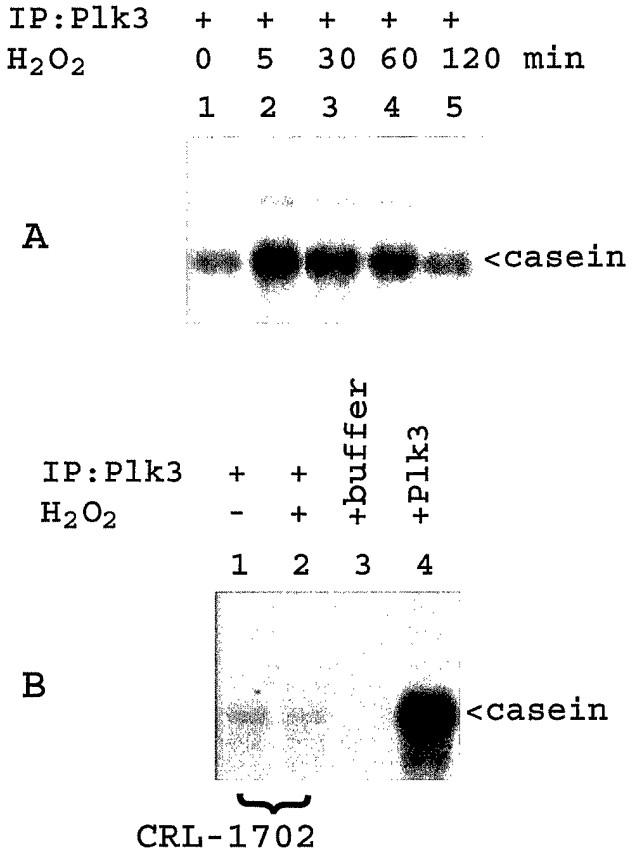

FIG. 3. **H_2O_2 activates Plk3 in an ATM-dependent manner.** *A*, A549 cells treated with H_2O_2 for the indicated times were immunoprecipitated with an antibody to Plk3. Plk3 immunoprecipitates were analyzed for *in vitro* kinase activity using casein as substrate. *B*, Plk3 immunoprecipitated from ATM-deficient cells (*CRL-1702*) treated with (*lane 2*) H_2O_2 for 30 min, as well as the untreated parental cells (*lane 1*), were analyzed for *in vitro* kinase activity. Vehicle (buffer, *lane 3*) or recombinant His_6-Plk3 (*lane 4*) was used as control for the kinase assay.

These observations suggest that p53 activation by ROS is at least in part dependent on ATM and/or ATR. To further confirm that ATM was important in mediating p53 phosphorylation by H_2O_2, we exposed ATM-deficient CRL-1702 cells to H_2O_2. Fig. 2*B* shows that in the ATM-deficient cells after H_2O_2 treatment enhancement in phosphorylation of p53 on neither serine 20 nor serine 15 was observed, indicating that ROS-induced phosphorylation and activation of p53 is ATM-dependent.

Our laboratory has been studying human Plk3, which is involved in regulating cell cycle progression (17, 25, 30). As an initial step to identify protein kinase(s) responsible for phosphorylation of p53 induced by ROS, we examined the possibility of Plk3 activation by H_2O_2, because Plk3 phosphorylates the same residue of Cdc25C (serine 216) as that targeted by Chk1 and Chk2. Chk1 and Chk2 are also reported to phosphorylate p53 on serine 20 (38, 39). A549 cells, expressing good levels of Plk3, were treated with ROS for various times. Plk3 immunoprecipitated from the treated cells was analyzed for its kinase activity using casein as substrate as described previously (25). Fig. 3*A* shows that compared with the control (*lane 1*) Plk3 kinase activity was rapidly activated in A549 cells (*lane 2*) and maintained for at least 1 h. To determine whether Plk3 activation was ATM-dependent, CRL-1702 cells treated with H_2O_2 were collected, and Plk3 immunoprecipitates were assayed for Plk3 kinase activity. Fig. 3*B* shows that whereas recombinant Plk3 phosphorylated casein effectively (*lane 4*), no difference in Plk3 kinase activity was detected between untreated control (*lane 1*) and H_2O_2-treated CRL-1702 cells (*lane 2*), suggesting that Plk3 activation also requires ATM. In addition, Plk3 activation was caffeine-sensitive because pretreatment of A549 cells with caffeine completely blocked activation of Plk3 by H_2O_2 (data not shown).

To determine the possibility that Plk3 was involved in mediating H_2O_2-induced p53 phosphorylation, we screened a dozen cell lines for Plk3 expression. We observed (Fig. 4*A*) that Daudi (B lymphoblastic leukemic cells with wild-type p53 (40)) did not express detectable levels of Plk3, whereas other tested cell lines expressed various levels of this protein. Further analysis with polymerase chain reaction confirmed that no Plk3 expression was detectable in Daudi cells (data not shown). To determine whether the absence of Plk3 expression affected p53 phosphorylation, we analyzed p53 phosphorylation on both serine 20 and serine 15 residues in Daudi cells exposed to H_2O_2. Fig. 4*B* shows that p53 phosphorylation on serine 15 is rapidly induced and maintained for at least 2 h in Daudi cells (*lanes 2–5*). In contrast, no serine 20 phosphorylation was observed. Chk2 is reported to phosphorylate p53 on serine 20 (39). Reprobing the same blot with antibody to Chk2 revealed that

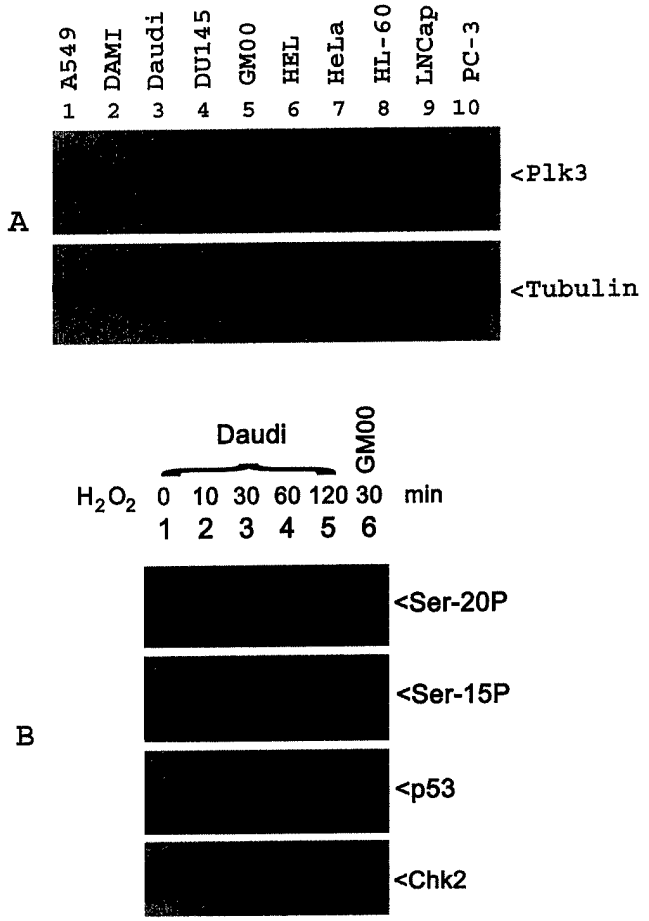

FIG. 4. **Absence of serine 20 phosphorylation in Daudi cells that express no Plk3.** *A*, equal amounts of protein lysates from the indicated cell lines were blotted for both Plk3 and α-tubulin expression. *B*, equal amounts of proteins from Daudi cells treated with H_2O_2 for the indicated times were blotted for p53 phosphorylation using phospho-specific antibody to serine 20 or serine 15 of p53 as well as all forms of p53 and Chk2. GM00637 cell lysates (*lane 6*) were used as a control.

Daudi cells expressed abundant Chk2 (Fig. 4*B*). These observations suggest that Plk3 is involved in regulating serine 20 phosphorylation of p53.

We next asked whether Plk3 directly phosphorylated p53. *In vitro* kinase assays showed (Fig. 5*A*) that recombinant histidine-tagged Plk3 (His_6-Plk3) phosphorylated GST-p53 (*lane 2*), as well as casein (*lane 1*), but not GST alone (*lane 5*), indicating that Plk3 targets the p53 moiety of GST-p53. A kinase-defective mutant of Plk3, His_6-$Plk3^{K52R}$, in which lysine 52 was replaced with arginine, did not significantly phosphorylate GST-p53 (Fig. 5*A*, *lane 3*). To further examine whether the serine 20 residue of p53 was a phosphorylation target of Plk3, we incubated GST-p53 with His_6-Plk3 or His6-$Plk3^{K52R}$ in the kinase buffer supplemented with ATP. *In vitro* phosphorylated GST-p53 samples, as well as nonphosphorylated GST-p53, were blotted for serine 20 phosphorylation. Fig. 5*B* shows that purified GST-p53 was not recognized by the antibody to serine 20-phosphorylated p53 (*lane 4*). However, when phosphorylated *in vitro* by His_6-Plk3, but not by His_6-$Plk3^{K52R}$, GST-p53 exhibited a strong phosphoserine 20 epitope (*lane 5*). Given that Plk3 kinase activity and serine 20 phosphorylation of p53 are induced by H_2O_2, these observations strongly suggest that serine 20 is an *in vivo* target of Plk3 during H_2O_2-induced stress response.

To explore the physical interaction between p53 and Plk3, we immunoprecipitated Plk3 from cells treated with or without H_2O_2, and Plk3 immunoprecipitates were then blotted for the

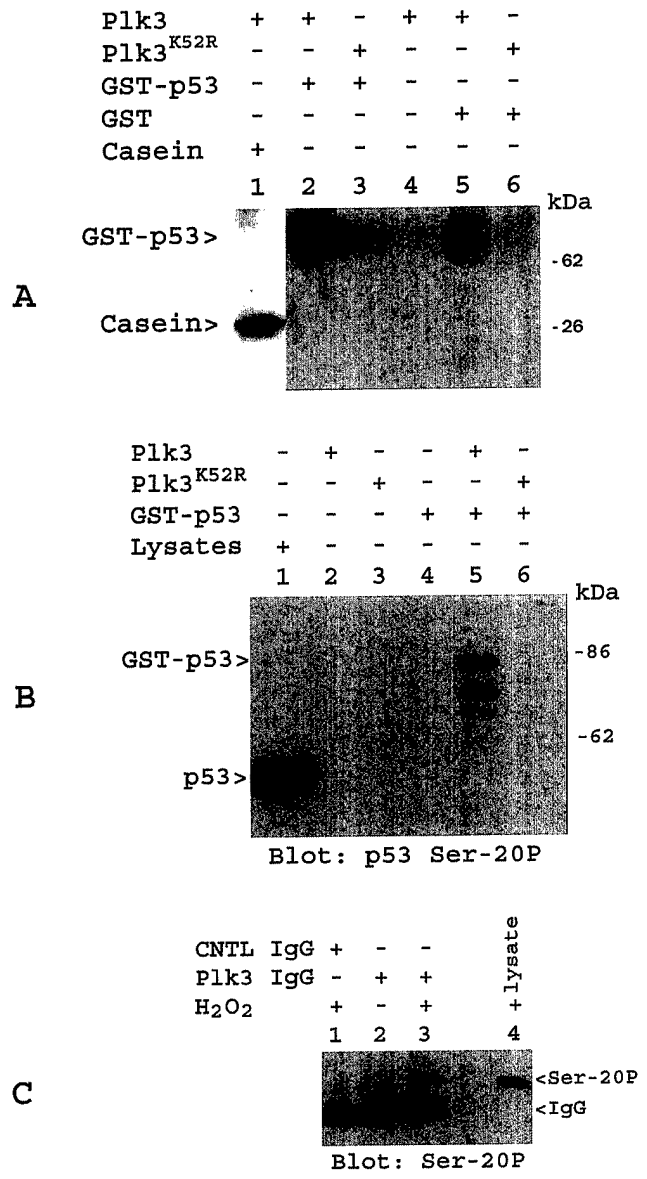

FIG. 5. **Plk3 interacts with and phosphorylates p53 *in vitro*.** *A*, purified GST-p53 was phosphorylated *in vitro* by His_6-Plk3 (*lane 2*) or His_6-$Plk3^{K52R}$ (*lane 3*) in the kinase buffer supplemented with [γ-^{32}P]ATP. GST and α-casein were used as negative and positive controls, respectively. After kinase reaction, samples were fractionated on SDS-polyacrylamide gel electrophoresis followed by autoradiography. *B*, GST-p53 was phosphorylated *in vitro* by His_6-Plk3 and His_6-$Plk3^{K52R}$ in the kinase buffer supplemented with "cold" ATP. The reaction samples and protein lysates from H_2O_2-treated GM00637 cells (*lane 1*) were then blotted with the antibody to phosphoserine 20 of p53. Partial degradation of GST-p53 was observed (*lane 5*). *C*, equal amounts of protein lysates from GM00637 cells treated with or without H_2O_2 were immunoprecipitated with the antibody to Plk3 or control IgGs. Immunoprecipitates were then blotted for serine 20-phosphorylated p53. GM00637 cell lysates were used as a positive control.

presence of serine 20-phosphorylated p53. Fig. 5*C* shows that neither the control IgGs appreciably precipitated serine 20-phosphorylated p53 from the H_2O_2-treated cells (*lane 1*) nor Plk3 antibody brought down the phospho-p53 from the untreated control cells (*lane 2*) However, Plk3 antibody precipitated p53 that was phosphorylated on serine 20 from cells treated with H_2O_2.

To further demonstrate that Plk3 regulated serine 20 phosphorylation of p53 *in vivo*, GM00637 cells were transfected with constructs expressing either Plk3 or $Plk3^{K52R}$. One day after transfection, both Plk3 proteins were expressed (Fig. 6*A*,

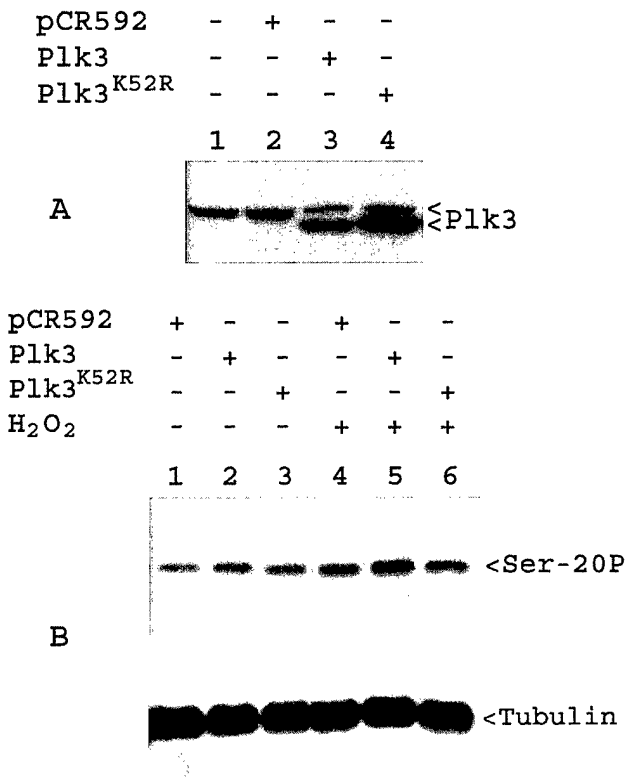

FIG. 6. **Plk3 regulates serine 20 phosphorylation of p53 *in vivo*.** *A*, GM00637 cells were transfected with constructs expressing Plk3 or $Plk3^{K52R}$ or with the vector alone. One day after transfection, cells were lysed, and equal amounts of proteins from the transfected cells were blotted with the antibody to Plk3. *B*, GM00637 cells transfected with various constructs as indicated were treated with or without H_2O_2 (20 μM) for 30 min. Equal amounts of proteins from various treatments were blotted with antibodies to serine 20-phosphorylated p53 or α-tubulin.

lanes 3 and *4*) at a level higher than the endogenous one (the band with a slower mobility). The fast mobility of both transfected Plk3 proteins was due to a short truncation at the amino terminus. Further analysis of the transfected cells showed (Fig. 6*B*) that no significant enhancement in serine 20 phosphorylation was detected when cells were transfected with either Plk3 (*lane 2*) or $Plk3^{K52R}$ (*lane 3*) compared with cells transfected with vector alone (*lane 1*). However, when Plk3-transfected cells were exposed to a low concentration of H_2O_2, a significant increase in serine 20 phosphorylation was detected (*lanes 2* and *5*). In contrast, no such enhancement in serine 20 phsophorylation was detected in cells transfected with $Plk3^{K52R}$ (*lanes 3* and *6*). These observations suggest that Plk3 needs to be activated by ROS before it can fully phosphorylate its physiological substrates.

DISCUSSION

The mechanism by which mammalian cells transmit signals in response to oxidative damage remains unclear. Here we report that ROS phosphorylates and activates p53 tumor suppressor protein. Consequences of p53 activation are either cell cycle arrest or apoptosis. We have observed that p53 activation in response to H_2O_2 treatment results in significant increase in expression of p21, but not of Bax (Fig. 1*B*), which is consistent with our observation that the concentration of H_2O_2 used in our experiments did not cause significant apoptosis of GM00637 cells (data not shown). However, we cannot exclude the possibility of Bax activation by post-translational mechanisms. Interestingly, it has been proposed that p53 may cause cell death by directly stimulating mitochondria to produce an excess amount of toxic ROS in some cells (33). Thus, a feedback loop between p53 and ROS may exist, which is presumably to amplify the stress signal, resulting in accelerated programmed cell death when damage caused by a genotoxic stress is beyond repair.

Recent advances indicate that reversible phosphorylation plays an important role in the DNA damage checkpoint activation. In fact, p53 is rapidly phosphorylated upon exposure of cells to IR or UV (6). Our current studies demonstrated that oxidative stress activates p53 also through phosphorylation on multiple residues. The kinetics of ROS-induced phosphorylation of p53 on various serine residues is apparently different (Fig. 1*A*), suggesting the involvement of several protein kinases. It is also likely that phosphorylation of certain residues may facilitate the subsequent phosphorylation of other residues. Consistent with the latter scenario, phopshorylation of threonine 18 by casein kinase II requires prior phosphorylation of serine 15 by ATM upon DNA damage (41).

Our current studies indicate that Plk3 is directly involved in H_2O_2-induced phosphorylation of p53 on the serine 20 residue. First, induction of both p53 phosphorylation and Plk3 kinase activity by H_2O_2 is ATM-dependent (Figs. 2*B* and 3*B*). Second, H_2O_2 does not induce serine 20 phosphorylation of p53 in Daudi cells that express Chk2 but no detectable levels of Plk3 (Fig. 4). Third, Plk3, but not $Plk3^{K52R}$, directly phosphorylates GST-p53 (but not GST alone) *in vitro*, and Plk3-phosphorylated GST-p53 contains a strong serine 20 epitope (Fig. 5). Fourth, Plk3 interacts with serine 20-phosporylated p53 when cells are exposed to H_2O_2 (Fig. 5*C*). Fifth, ectopic expression of Plk3, but not the kinase-defective mutant $Plk3^{K52R}$, results in significantly enhanced phosphorylation of p53 on serine 20 after H_2O_2 treatment.

Our studies, together with previous observations (30), suggest that Plk3 may act in parallel with Chk1 and Chk2, downstream of ATM or ATR. Plk3 may preferentially transduce signals generated by a specific genotoxic stress such as H_2O_2, just as Chk1 and Chk2 are differentially activated by UV radiation and IR, respectively (6). The observation that serine 20 phosphorylation of p53 was not induced by H_2O_2 in Daudi cells that express abundant Chk2 but no detectable Plk3 supports this notion. On the other hand, given that Cdc5 acts downstream of Rad53 in yeast (26), it is also possible that Plk3 may lie downstream of Chk2 (and/or Chk1). Plk3 may integrate the signals from ATM-Chk2 and ATR-Chk1 and induce cell cycle arrest or apoptosis by phosphorylating either Cdc25C on serine 216 or p53 on serine 20. Consistent with the latter scenario, Plk3 is activated by IR-mimetic drug adriamycin and UV radiation (data not shown) in addition to H_2O_2.

Acknowledgments—We are grateful to Jessica Chen for technical assistance. We also thank Intisar Husain for Plk3 and $Plk3^{K52R}$ expression construct.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 46, Issue of November 16, pp. 43305–43312, 2001
Printed in U.S.A.

Plk3 Functionally Links DNA Damage to Cell Cycle Arrest and Apoptosis at Least in Part via the p53 Pathway*

Received for publication, June 29, 2001, and in revised form, September 7, 2001
Published, JBC Papers in Press, September 10, 2001, DOI 10.1074/jbc.M106050200

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Polo-like kinase 3 (Plk3, previously termed Prk) contributes to regulation of M phase of the cell cycle (Ouyang, B., Pan, H., Lu, L., Li, J., Stambrook, P., Li, B., and Dai, W. (1997) *J. Biol. Chem.* 272, 28646–28651). Plk3 physically interacts with Cdc25C and phosphorylates this protein phosphatase predominantly on serine 216 (Ouyang, B., Li, W., Pan, H., Meadows, J., Hoffmann, I., and Dai, W. (1999) *Oncogene* 18, 6029–6036), suggesting that the role of Plk3 in mitosis is mediated, at least in part, through direct regulation of Cdc25C. Here we show that ectopic expression of a kinase-active Plk3 (Plk3-A) induced apoptosis. In response to DNA damage, the kinase activity of Plk3 was rapidly increased in an ATM-dependent manner, whereas that of Plk1 was markedly inhibited. Recombinant Plk3 phosphorylated *in vitro* a glutathione *S*-transferase fusion protein containing p53, but not glutathione *S*-transferase alone. Recombinant Plk1 also phosphorylated p53 but on residues that differed from those targeted by Plk3. Co-immunoprecipitation and pull-down assays demonstrated that Plk3 physically interacted with p53 and that this interaction was enhanced upon DNA damage. *In vitro* kinase assays followed by immunoblotting showed that serine 20 of p53 was a target of Plk3. Furthermore, expression of a kinase-defective Plk3 mutant ($Plk3^{K52R}$) resulted in significant reduction of p53 phosphorylation on serine 20, which was correlated with a decrease in the expression of p21 and with a concomitant increase in cell proliferation. These results strongly suggest that Plk3 functionally links DNA damage to cell cycle arrest and apoptosis via the p53 pathway.

In mammals, DNA damage induced by ionizing radiation or UV light is detected by two serine/threonine kinases known as "mutated in ataxia telangiectasia" (ATM)[1] and "ATM-related" (ATR) (3–5). Depending on the extent of DNA damage, cells either undergo cell cycle arrest or initiate apoptosis, responses that are at least partly mediated by p53 (4–6). Activated ATM and ATR phosphorylate p53 on serine 15 (7–9), thereby contributing to the activation of the tumor suppressor protein. The kinases Chk1 and Chk2, which act downstream of ATR and ATM, respectively (10–12), are reported to phosphorylate p53 *in vitro* on serine 20 (13–14); this residue is located within the domain of the protein that interacts with HDM2, resulting in stabilization of the normally short lived p53 protein in response to DNA damage (15).

Polo family kinases also play a role in the DNA damage response (11, 16, 18). Cdc5, a polo homolog in budding yeast, is modified in its mobility on denaturing gels in response to DNA damage, and this modification is dependent on MEC1, Rad53 (a Chk homolog), and Rad9 (16). In addition, a functionally defective Cdc5 mutant suppresses a Rad53 checkpoint defect, whereas overexpression of Cdc5 overrides checkpoint-induced cell cycle arrest (11). Moreover, DNA damage appears to interfere with the activation of Plk1 in mammals, resulting in down-regulation of the kinase activity of this protein (18). On the other hand, expression of Plk1 mutants that are nonresponsive to DNA damage overrides G_2 arrest. Mammalian Plk3 is a structural homolog of Plk1 (19), and its expression is down-regulated in several types of cancer (19, 20). Both Plk1 and Plk3 can rescue the temperature-sensitive phenotype of yeast Cdc5 mutants (1, 21). However, evidence indicates that Plk3 functions differently from Plk1 in regulation of cell proliferation and oncogenesis in mammalian cells (1, 2, 20).

We have previously shown that Plk3 phosphorylates Cdc25C on serine 216 (2), a site that is also targeted by Chk1 and Chk2 (10, 22). Phosphorylation of serine 216 of Cdc25C is inhibitory, which is due to sequestration of the protein phosphatase in the cytoplasm by 14-3-3 protein (24). In this report, we have provided evidence indicating that Plk3 is involved in DNA damage checkpoint response and that it may target p53 *in vivo* through regulation of phosphorylation on serine 20. A model is proposed that explains the mechanism of action of Plk3 during genotoxic stress-induced activation of the DNA damage checkpoint, which results in cell cycle arrest and/or apoptosis.

EXPERIMENTAL PROCEDURES

Immunoblotting and Pull-down Assays—Various cell lines were obtained from ATCC, except for the GM00637 cell line, which was originally from the Coriell Institute for Medical Research. Cells treated with adriamycin (100 μM) for 30 min were collected and lysed (1). Equal amounts (50 μg) of protein lysates from various cell lines were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies to human Plk3 (Pharmingen) (2, 25), α-tubulin (Sigma), and p53 (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Pharmingen). Recombinant His_6-Plk3 expressed with the use of a baculoviral expression system as described (2) was affinity-purified with and subsequently conjugated to Ni^{2+}-NTA resin (Qiagen). Plk3-conjugated Ni^{2+}-NTA resin or the resin alone was incubated for 3 h at 4 °C with lysates (1 mg of protein) of GM00637 cells that had been pretreated for 30 min with adriamycin. After washing of the resin, bound proteins were eluted and subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis with antibodies to p53 or serine 20-phospho-

* This work was supported in part by Public Health Service Award RO1-74229 and a Department of Defense award (to W. D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ATM, mutated in ataxia telagiectasia; ATR, ATM-related; GST, glutathione *S*-transferase; NTA, nitrilotriacetic acid.

rylated p53 (New England Biolabs). The p53 antigens were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Each experiment was repeated at least three times.

Co-immunoprecipitation Analysis—GM00637 cell lysates (1 mg of protein) were incubated for 30 min at room temperature in a total volume of 500 μl with of 20 μl of protein A/G-agarose bead slurry (Santa Cruz Biotechnology). After removal of the beads, the supernatant was supplemented with either rabbit polyclonal (Pharmingen) or mouse monoclonal antibodies to p53 or with mouse monoclonal antibodies to CD45 (Pharmingen), followed by incubation for an additional 2 h at room temperature or overnight at 4 °C. Protein A/G-agarose beads (20 μl) were then added to each immunoprecipitation mixture, and the incubation was continued for 1 h at room temperature. Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to immunoblot analysis with a monoclonal antibody to Plk3.

Immunocomplex Kinase Assays—Immunocomplex kinase assays were performed essentially as described (1). In brief, A549 or ATM-deficient (ATCC number CRL-7201) (23) cells were exposed to adriamycin for various times, lysed, and subjected to immunoprecipitation with antibodies to Plk3 or to Plk1 (Zymed Laboratories Inc.). The resulting precipitates were resuspended in kinase buffer (10 mM Hepes-NaOH (pH 7.4), 10 μM $MnCl_2$, 5 mM $MgCl_2$), and the kinase reaction was initiated by the addition of [γ-^{32}P]ATP (2 μCi) (Amersham Pharmacia Biotech) and either α-casein (Sigma), GST-p53, or GST. After incubation for 30 min at 37 °C, the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The phosphorylated α-casein was quantified by densitometric scanning. Recombinant His_6-Plk3, His_6-Plk1, and His_6-Plk3^{K52R} were also assayed for kinase activity as controls. In some kinase assays, GST-p53 or GST-p53^{S20A} was incubated with His_6-Plk3 in the kinase buffer supplemented with "cold" ATP. After reaction, GST-p53 and GST-p53^{S20A} were blotted for serine 20 phosphorylation. Each assay was repeated for at least three times.

Phosphopeptide Mapping—Tryptic peptide mapping was performed essentially as described (2, 26). In brief, ^{32}P-labeled p53 was excised from dried SDS-polyacrylamide gels and eluted into extraction buffer (50 mM NH_4HCO_3, 0.1% SDS, 1% 2-mercaptoethanol). After removal of debris by centrifugation, the eluted protein was supplemented with 100 μg of acetylated bovine serum albumin (Sigma) and then precipitated by the addition of trichloroacetic acid. The protein precipitate was recovered by centrifugation, washed once with ethanol, dissolved in performic acid (98% formic acid, 30% H_2O_2; 9:1 (v/v)), and lyophilized. The dried protein was resuspended in 50 mM NH_4HCO_3 (pH 8.0) and subjected to digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma). The resulting peptides were applied to thin layer chromatography plates and fractionated by electrophoresis followed by chromatography as described (26) with a Multiphor II (Amersham Pharmacia Biotech) apparatus. The plates were then air-dried and subjected to autoradiography. The mapping experiment was repeated at least three times.

Transient Transfection—HeLa cells were transfected, using the LipofectAMINE method (Life Technologies, Inc.), with pCR259Plk3^{K52R} (1, 20), pcDNA3-p53, pcDNA3-p53^{S20A}, or the empty vectors (Invitrogen). The serine 20 mutant (serine 20 was replaced with alanine) of p53 was obtained via site-directed mutagenesis using a kit purchased from Stratagene according to the protocol provided by the supplier. One day after transfection, cells were treated with or without 100 μM adriamycin for 1 h. Cell lysates were prepared and blotted for Plk3, p53, or p21 expression. Two different antibodies to p53 were used. One was from Santa Cruz Biotechnology (DO-1, the recognition epitope of which was between residues 11 and 25), and the second one was from Pharmingen (G59-12). To determine the effect of p53 or Plk3^{K52R} on overall cell growth, HeLa cells transfected with p53, p53^{S20A}, and/or Plk3^{K52R} were cultured in medium containing G418 (600 μg/ml) and maintained for 2 weeks. Colonies formed were visualized after staining with 0.125% crystal violet solution. As an alternative method to determine cell proliferation, triplicate transfected cells that were maintained in G418-containing medium (600 μg/ml) for 1 week were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as described (27). ODs at 570 nm were determined for each sample. Each transfection experiment was repeated at least three times.

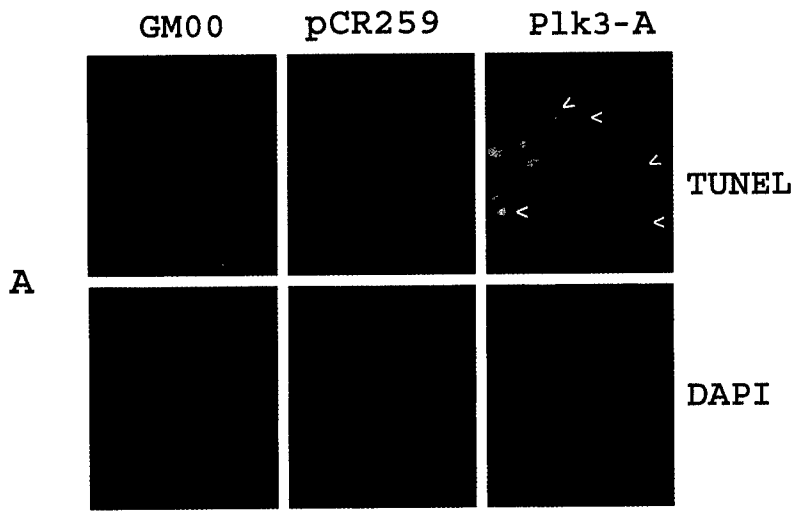
DNA Fragment End Labeling Assay—End labeling of DNA fragments was performed using a kit purchased from Oncogene Research Products (Boston, MA) according to the protocol provided by the manufacturer. Briefly, GM00637 cells cultured on cover slides were transfected with Plk3-A expression construct or with the vector alone for 18 h. The transfected cells as well as the untransfected parental cells were washed once with phosphate-buffered saline and then sequentially fixed in 4% paraformaldehyde and 80% ethanol for 10 min each. The fixed cells were rehydrated in Tris-buffered saline for 10 min and then treated with proteinase K (2 mg/ml) for 4 min. The treated cells were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase at 37 °C in a humid chamber for 90 min. After reaction, cells were stained with 4′,6-diamidino-2-phenylindole (1 mg/ml) for 5 min. Fluorescence microscopy was performed on an Olympus AT70 microscope (Spot Diagnostic Instrument), and images were captured using a digital camera using Image System Spot RT software. GM00637 cells transfected with Plk3-A, Plk3^{K52R}, or the vector alone for 1 day were also collected for genomic DNA isolation. The isolated DNA (10 μg/lane) was analyzed on agarose gels.

RESULTS

Plk3 is a structurally conserved protein serine/threonine kinase playing an important role in the regulation of M phase function (1). A recent study indicates that overexpression of Plk3 induces chromatin condensation and programmed cell death (25). To confirm the role of Plk3 in induction of apoptosis, we transfected GM00637 fibroblast cells with a plasmid construct expressing kinase-active Plk3 (Plk3-A) (1, 2). DNA fragmentation analyses (Fig. 1) revealed that transfection of Plk3-A, but not the vector alone, induced significant DNA fragmentation, indicative of apoptosis. Transfection of Plk3^{K52R} in which lysine 52 was replaced with arginine also induced DNA fragmentation, albeit it was less significant than that of Plk3-A (Fig. 1*B*).

Recent studies have shown that phosphorylation of Cdc25C is inhibitory (22, 24). The observations that Plk3 phosphorylates serine 216 of Cdc25C (2) and that polo family kinases contribute to regulation of the DNA damage checkpoint (11, 16, 18) prompted us to investigate whether the kinase activity of Plk3 is affected in cells subjected to DNA damage. Immunocomplex kinase assays with α-casein as substrate revealed that the kinase activity of Plk3 was increased more than 10-fold by exposure of A549 cells to the DNA-damaging agent adriamycin (Fig. 2, *A* and *B*). A549 cells were used because they expressed good levels of Plk3. The activation of Plk3 in response to other genotoxic stresses such as UV or H_2O_2 was also detected (data not shown). Immunoblot analysis indicated (Fig. 2*A*) that Plk3 antigen was not increased upon adriamycin treatment, suggesting that the increase of Plk3 kinase activity was due to a post-translational mechanism(s). Given that the kinase activity of Plk1 has been shown to be down-regulated during activation of the DNA damage checkpoint (18) and that the antibody to Plk3 used for our immunocomplex kinase assay did not cross-react with human Plk1 (Fig. 2*C*), we measured the kinase activities of both Plk1 and Plk3 in the same A549 cells treated with adriamycin. Whereas little endogenous Plk3 kinase activity was detected under control conditions (Fig. 2*D*, *lane 1*), activation of Plk3 was apparent 10 min after exposure of the cells to adriamycin; in contrast, Plk1 was constitutively active under basal conditions (Fig. 2*D*, *lane 4*), and its activity was markedly inhibited in response to adriamycin treatment (*lanes 5* and *6*). Thus, Plk1 and Plk3 appear to be differentially regulated in response to DNA damage.

To examine whether the DNA damage-induced activation of Plk3 is dependent on ATM, we exposed A549 cells that had been pretreated with caffeine, which inhibits the kinase activities of ATM and ATR (28), to adriamycin. Caffeine not only blocked the activation of Plk3 by adriamycin but also inhibited the basal kinase activity of this protein (Fig. 3, *A* and *B*). To further confirm the dependence of Plk3 activation on ATM, we analyzed the Plk3 kinase activity in an ATM-deficient cell line (ATCC number CRL-7201) that had been treated with adriamycin for various times. No increase in Plk3 activity upon DNA damage was detected in the ATM-deficient cells (Fig. 3, *C* and *D*, *lanes 2–4*).



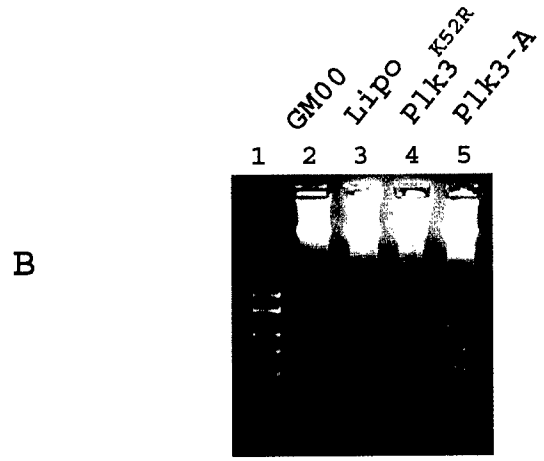


FIG. 1. **Ectopic expression of Plk3 induces apoptosis.** GM00637 cells transfected with the Plk3-A expression construct or the vector were analyzed for DNA fragmentation using a DNA fragment end labeling (FragEL™) detection kit as described under "Experimental Procedures" (*A*) or via agarose gel electrophoresis (*B*). Untransfected parental cells were also used for analyses. Representative results from three similar experiments were presented.

In addition to their reported phosphorylation of p53 on serine 20 (14, 29, 30), Chk1 and Chk2 phosphorylate serine 216 of Cdc25C (11, 22), phosphorylation of which is thought to be partly responsible for G_2 arrest during activation of the DNA damage checkpoint. Given that Plk3 phosphorylates the same residue of Cdc25C (serine 216) as that targeted by Chk1 and Chk2, we investigated whether Plk3 also phosphorylates p53 *in vitro*. Recombinant histidine-tagged Plk3-A (His_6-Plk3-A) phosphorylated GST-p53 (Fig. 4*A*, *lane 2*) but not GST alone (*lane 5*), indicating that Plk3 targets the p53 moiety of GST-p53. $Plk3^{K52R}$ phosphorylated GST-p53 to a greatly reduced extent (Fig. 4*A*, *lane 3*) compared with that observed with Plk3-A (*lane 2*). Recombinant (His_6)-Plk1 also phosphorylated GST-p53 (Fig. 4*A*, *lane 9*) but not GST alone (data not shown). Unlike His_6-Plk3, His_6-Plk1 exhibited a high level of autophosphorylation activity (Fig. 4*A*, *lane 9*).

The biological functions of mammalian Plk3 and Plk1 appear to differ (2, 19, 20, 32, 33), although both mammalian proteins complement Cdc5 temperature-sensitive mutants of budding yeast (1, 21). Overexpression of murine Plk1 results in oncogenic transformation (33), whereas ectopic expression of human Plk3 inhibits cell growth by inducing apoptosis (25). Thus, our observation that both Plk3 and Plk1 phosphorylate p53 suggested that these two kinases may target different residues of the tumor suppressor protein. Whereas p53 phosphorylated by Plk3 yielded two major phosphopeptides in phosphopeptide mapping analysis, p53 phosphorylated by Plk1 yielded four major phosphopeptides (Fig. 4*B*). Further analysis of the phosphopeptide maps indicated that peptides a and e were specific to Plk3-phosphorylated p53 and that peptides b, c, and d were unique to Plk1-phosphorylated p53.

Our observation that Plk3 phosphorylates p53 *in vitro* suggested that the two proteins might physically interact *in vivo*. Co-immunoprecipitation experiments (Fig. 5*A*) revealed that both antibodies (from two different sources) to p53, but not control antibodies to CD45, precipitated Plk3 from GM00637 cell lysates. Furthermore, Ni^{2+}-NTA resin conjugated with His_6-Plk3, but not Ni^{2+}-NTA resin alone, precipitated p53 from the cell lysates (Fig. 5*B*). The amount of p53 precipitated by the His_6-Plk3-conjugated resin was markedly increased by prior exposure of the cells to adriamycin (Fig. 5*B*, *lanes 3* and *4*), although treatment with this drug for 30 min did not affect the total amount of p53 present in the cells (*lanes 5* and *6*). Proteins eluted from both His_6-Plk3-conjugated resin and control resin were also blotted for the presence of phospho-p53. Fig. 5*B* shows that His_6-Plk3 resin precipitated serine 20-phosphorylated p53 and that the amount of the phosphorylated p53 pulled down by His_6-Plk3 was significantly increased following adriamycin treatment. These results thus suggest that activation of p53 promotes its interaction with Plk3.

Chk1 and Chk2, as well as Plk3, phosphorylate Cdc25C in serine 216 (2, 10, 22). The direct interaction of Plk3 with p53 and the observation that Chk1 and Chk2 also phosphorylate p53 on serine 20 prompted us to examine whether the serine 20 residue of p53 was a phosphorylation target of Plk3. *In vitro* kinase assays followed by immunoblotting showed (Fig. 5*C*, *lane 2*) that purified GST-p53 was not recognized by an antibody to serine 20-phosphorylated p53. However, GST-p53 phos-

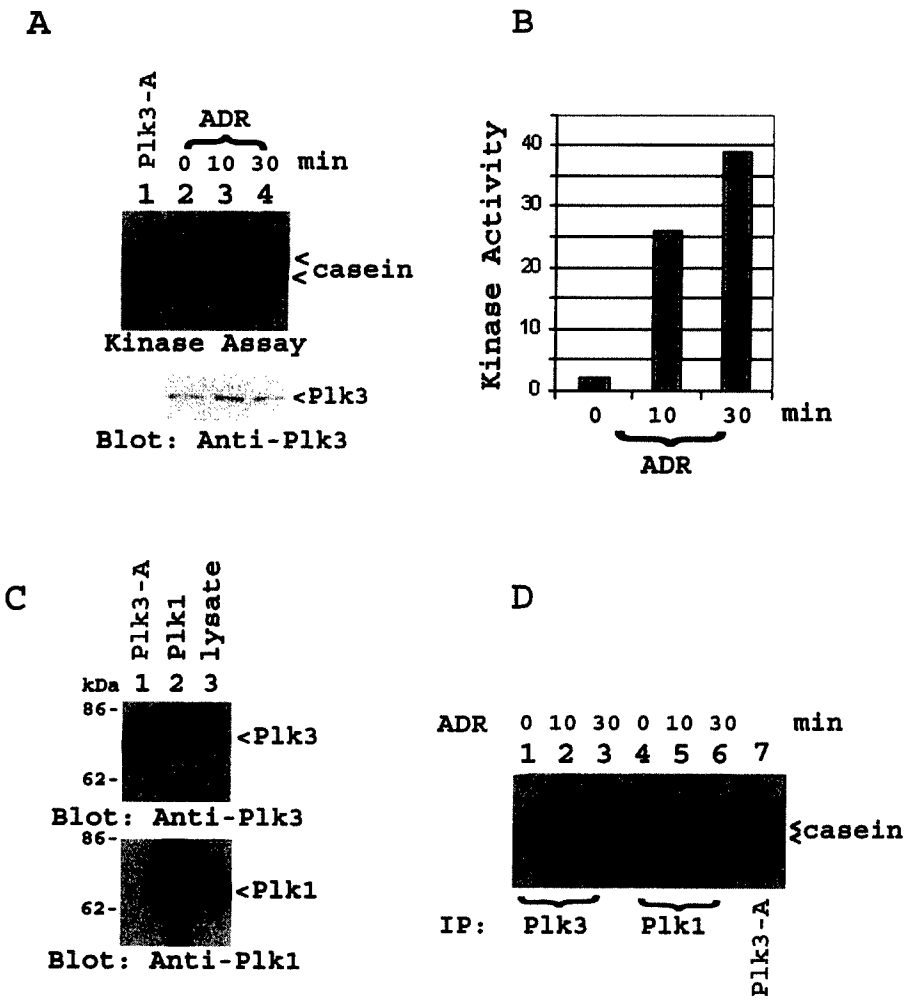


FIG. 2. **Activation of Plk3 in response to DNA damage.** *A*, A549 cells were incubated with 100 μM adriamycin (*ADR*) for the indicated times, after which cells were lysed and subjected to immunoprecipitation (*IP*) with antibodies to Plk3. The resulting precipitates were then assayed for kinase activity with α-casein as substrate. Recombinant His_6-Plk3-A (Plk3-A) was used as a positive control for *in vitro* assay of kinase activity. The *arrowheads* indicate phosphorylated α-casein. The same A549 cell lysate samples were also blotted with antibody to Plk3. *B*, quantification of Plk3 kinase activity as shown in *A*. The kinase units are arbitrary. *C*, duplicate protein blots with purified recombinant His_6-Plk3-A and Plk1, as well as A549 cell lysates, were probed with antibodies to Plk3 and Plk1, respectively. Recombinant Plk1 migrates slightly slower than the cellular one due to addition of a FLAG tag at the amino terminus. *D*, A549 cells were incubated with 100 μM adriamycin for the indicated times, after which cells were lysed and subjected to IP with antibodies to either Plk3 or Plk1, as indicated. The resulting precipitates were assayed for kinase activity. His_6-Plk3-A (*lane 7*) was used as a control for the kinase assay. Presented results were representative of at least three similar experiments.

phorylated *in vitro* by Plk3 exhibited a strong phosphoserine 20 epitope (*lane 3*). Furthermore, when an equal amount of GST-$p53^{S20A}$, a mutant in which serine 20 was replaced with alanine, was used as an *in vitro* substrate, no phosphoserine 20 epitope was detected (Fig. 5*C*, *lane 4*). Given that p53 serine 20 phosphorylation and Plk3 activation are induced by a variety of genome toxic stresses, these observations suggest that the serine 20 residue may be a direct *in vivo* target of Plk3.

To establish Plk3 serine 20 phosphorylation of p53 *in vivo*, we transfected both p53 and $p53^{S20A}$ expression plasmids into HeLa cells, which constitutively express Plk3 but not p53. Western blot analysis showed (Fig. 6*A*) that transfected p53, but not $p53^{S20A}$ mutant protein, was constitutively phosphorylated on serine 20. DNA damage induced by adriamycin significantly enhanced serine 20 phosphorylation of the transfected p53, indicating that the pathway leading to p53 serine 20 phosphorylation was intact, albeit endogenous p53 was inactivated in this tumor cell line.

To determine the effect of change in Plk3 activity on p53 phosphorylation, HeLa cells were cotransfected with plasmid constructs expressing p53 and $Plk3^{K52R}$. Western blot analysis showed (Fig. 6*B*) that p53 was expressed and phosphorylated on serine 20 and that expression of $Plk3^{K52R}$ significantly reduced the level of p53 phosphorylation on serine 20 (*lane 6*). To determine the consequence of serine 20 phosphorylation of p53 on its target gene expression, we measured p21 level in cells expressing p53 and/or the Plk3 dominant mutant. We have consistently observed (Fig. 6*B*) that while little p21 was detected in parental HeLa cells or cells transfected with vectors alone (*lanes 1* and *2*), ectopic expression of p53 greatly induced p21 expression (*lane 3*). Expression of $p53^{S20A}$ also induced p21 expression that was about 25% of that induced by wild-type p53 (*lane 4*). Further, expression of the $Plk3^{K52R}$ greatly reduced the ability of p53 to induce p21 expression (compare *lanes 3* and *6*), which was consistent with the reduced serine 20 phosphorylation of p53 as well as p53 protein levels. These observations thus clearly demonstrate that phosphorylation of p53 on serine 20 plays a significant role in activating p21 expression.

We then determined the colony-forming efficiency of HeLa cells that were cotransfected with $Plk3^{K52R}$ and p53. Fig. 7*A* shows that p53 suppressed colony formation of HeLa cells. $Plk3^{K52R}$ alone also suppressed the colony formation. However, when cotransfected with p53, $Plk3^{K52R}$ significantly blocked p53-mediated suppression of colony formation of HeLa cells. Independent assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method confirmed that $Plk3^{K52R}$ effectively blocked the ability of p53 to inhibit HeLa cell proliferation (Fig. 7*B*). It is interesting that $Plk3^{K52R}$ also had an effect on $p53^{S20A}$ in terms of cell growth. This effect was likely to be mediated by inhibition of phosphorylation of other serine/threonine sites by $Plk3^{K52R}$ because Plk3 phosphorylates p53 on multiple sites *in vitro* (Fig. 4*B*).

DISCUSSION

Our results demonstrate that Plk3 contributes to DNA damage checkpoint activation, which is at least partly mediated by regulating phosphorylation of p53. The adriamycin-induced activation of Plk3 was caffeine-sensitive and not observed in

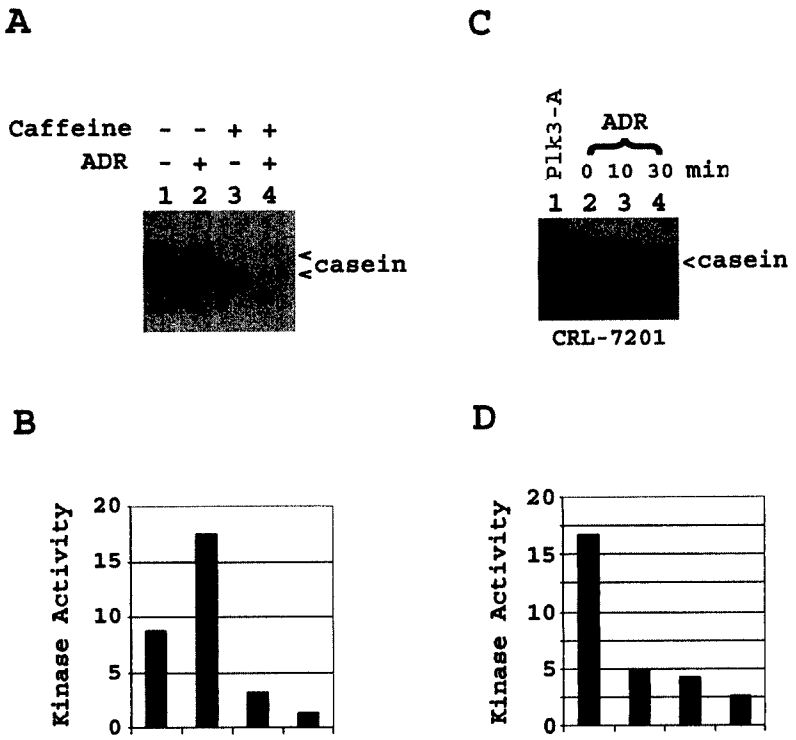


FIG. 3. **Plk3 activation is ATM-dependent.** *A*, A549 cells were preincubated with vehicle (medium) or 5 μM caffeine for 2 h and then incubated with 100 μM adriamycin for 30 min. Plk3 immunoprecipitates were then prepared and assayed for kinase activity. *B*, quantification of Plk3 kinase activity as shown in *A*. Units are arbitrary. *C*, an ATM-deficient cell line CRL-7201 was treated with 100 μM adriamycin for the indicated times, and Plk3 proteins immunoprecipitated from the treated cells were assayed for kinase activity. His6-Plk3-A was used as a control (*lane 1*). *D*, quantification of Plk3 kinase activity as shown in *C*. Units are arbitrary.

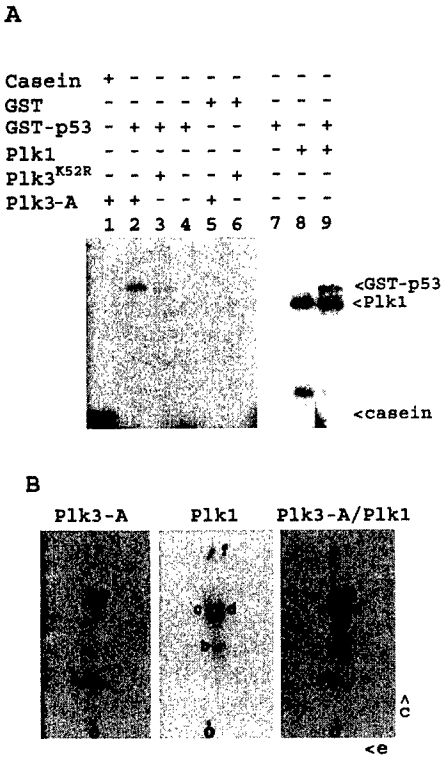


FIG. 4. **Phosphorylation of p53 on different residues by Plk3-A and Plk1.** *A*, recombinant His_6-Plk3-A, His_6-$Plk3^{K52R}$, and Plk1 were assayed for kinase activity with GST-p53 as substrate. α-casein and GST were used as positive and negative control substrates, respectively. *B*, two-dimensional analysis of ^{32}P-labeled tryptic peptides derived from GST-p53 phosphorylated *in vitro* by either His_6-Plk3-A (*left panel*) or His_6-Plk1 (*middle panel*), or mixed samples (*right panel*). The directions of electrophoresis (*e*) and chromatography (*c*) are indicated, as is the origin (*o*).

ATM-deficient cells, indicating the participation of ATM. Phosphorylation is thought to play an important role in regulation of the stability and activity of p53. This study suggests that serine 20 of p53 may be an *in vivo* target of Plk3 during DNA damage checkpoint activation because recombinant Plk3 phosphorylates p53 *in vitro*, resulting in a strong phosphoserine 20 epitope (Fig. 5*C*) and because it also interacts with serine 20-phosphorylated p53. Furthermore, expression of $Plk3^{K52R}$ in HeLa cells results in decreased phosphorylation of cotransfected p53 on serine 20, which was correlated with a significant reduction in p21 expression (Fig. 6*B*) with a concomitant increase in cell proliferation (Fig. 7).

Together with previous observations (2), our data suggest a simple and somewhat redundant set of mechanisms for DNA damage-induced signal transduction between ATM or ATR and effector molecules, resulting in cell cycle arrest and apoptosis (Fig. 8). According to this model, Plk3 may act in parallel with Chk1 and Chk2, downstream of ATM or ATR. It is possible that Plk3 preferentially transduces signals generated by a specific genotoxic stress, just as Chk1 and Chk2 are differentially activated by UV radiation and ionizing radiation, respectively (6). Alternatively, Plk3 may be activated by Chk2 (and/or Chk1), given that Cdc5 acts downstream of Rad53 in yeast (16). Plk3 may thus integrate the signals from ATM-Chk2 and ATR-Chk1 and induce cell cycle arrest or apoptosis by phosphorylating both p53 and Cdc25C. Consistent with this second scenario, Plk3 is activated by the ionizing radiation-mimetic drug adriamycin, UV radiation, and oxidative stress.[2]

A third possibility also exists to explain our observations; namely Chk1 and Chk2 preferentially target serine 20 of p53. In this case, p53, phosphorylated on serine 20 by Chk1 and Chk2, interacts with high affinity to Plk3, resulting in phosphorylation of p53 on additional sites. This would also place Plk3 downstream of Chk1/Chk2 in the DNA damage checkpoint pathway. It is possible that phosphorylation of p53 on other sites enforces the DNA damage checkpoint, but is not absolutely required. Although we observed that $Plk3^{K52R}$ expression reduced levels of serine 20 phosphorylation of p53, overexpression of this mutant protein may result in more efficient interaction with p53, potentially blocking access of p53 to

[2] S. Xie, H. Wu, Q. Wang, and W. Dai, unpublished data.

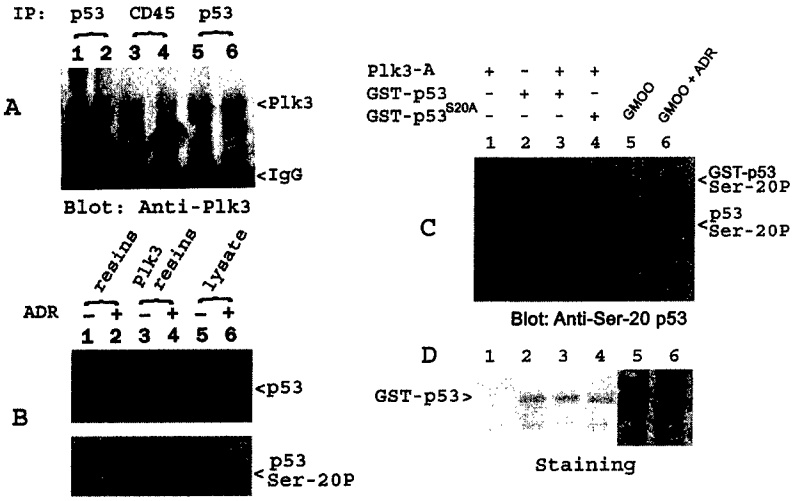
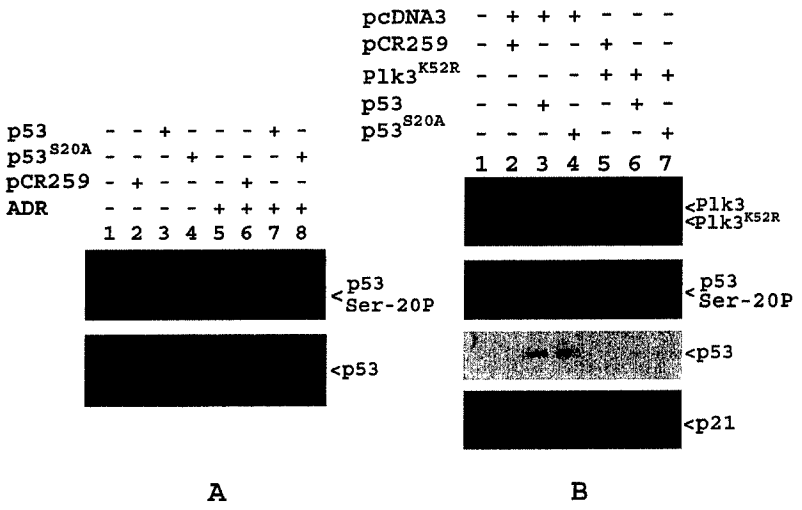


FIG. 5. **Plk3 interacts with p53 and phosphorylates the tumor suppressor protein on serine 20.** *A*, lysates from GM00637 cells were subjected to immunoprecipitation (*IP*) with polyclonal (*lanes 1* and *2*) or monoclonal (*lanes 5* and *6*) antibodies to p53 or with monoclonal antibodies to CD45 as a negative control (*lanes 3* and *4*). The positions of bands corresponding to Plk3 and immunoglobulin G (IgG) are indicated. *B*, His_6-Plk3-A immobilized on Ni^{2+}-NTA resin (*lanes 3* and *4*) or resin alone (*lanes 1* and *2*) was incubated with lysates of GM00637 cells that had been pretreated for 30 min with (*lanes 2* and *4*) or without (*lanes 1* and *3*) adriamycin. Proteins that interacted specifically with the His_6-Plk3-A-conjugated resin or resin alone were eluted and subjected to immunoblot analysis with antibodies to p53 or serine 20-phosphorylated p53. 50 μg of the cell lysates were also directly subjected to immunoblot analysis (*lanes 5* and *6*). *C*, GST-p53 and GST-$p53^{S20A}$ were incubated *in vitro* in the kinase buffer with His_6-Plk3-A, and the phosphorylated proteins (*lanes 3* and *4*) as well as the nonphosphorylated GST-p53 (*lane 2*) were subject to immunoblot analysis with antibodies to serine 20-phosphorylated p53. Lysates from GM00637 cells treated with or without adriamycin were used as controls for immunoblotting. *D*, the blot as shown in *C* was stained with Coomassie Blue as a loading control. Some degradations of GST-p53 were observed. The results are representative of three similar experiments.

FIG. 6. *A*, HeLa cells transfected with p53, $p53^{S20A}$, or vector were treated with or without adriamycin for 30 min. Cell lysates were then analyzed for serine 20-phosphorylated p53 as well as total p53. The antibody to p53 recognized an epitope between residues 11 and 25 (Santa Cruz Biotechnology). Thus, serine 20 mutation significantly compromised the detection. *B*, HeLa cells were cotransfected with various expression plasmids for 24 h as indicated. Cell lysates were collected and blotted for Plk3, serine 20-phosphorylated p53, total p53, and p21. Recognition of total p53 by the p53 antibody (Pharmingen) was not affected by the serine 20 mutation. The results were representative of three independent experiments.



Chk1 or Chk2. Therefore, the *in vivo* role of Plk3 in regulating serine 20 phosphorylation remains to be established, which would require obtaining Plk3 null cells.

Several other residues of p53 are potential sites of phosphorylation by Plk3. Phosphorylation of threonine 18 is induced by DNA damage, and a casein kinase 1-like enzyme is thought to phosphorylate this residue in A549 cells (31). However, the identity of this casein kinase 1-like enzyme is unknown. Given that Plk3, a casein-phosphorylating kinase, is activated by DNA damage, Plk3 may also target threonine 18 of p53. Moreover, given the apoptosis-inducing function of Plk3 (Fig. 1) and the observations both that residues 43–63 of p53 are necessary for induction of apoptosis by p53 (23) and that serine 46 phosphorylation is important for the transactivation of apoptosis-inducing genes by p53 (17), serine 46 may be another potential site of phosphorylation of p53 by Plk3.

Overexpression of Plk3-A induced a significant level of DNA fragmentation in GM00637 cells (Fig. 1), which is consistent with an early finding that Plk3 induces chromatin condensation and apoptosis (25). Given that p53 is an *in vitro* substrate of Plk3-A, it is reasonable to predict that Plk3-induced apoptosis may be mediated through phosphorylation and activation of p53. However, we were unable to detect enhanced serine 20 phosphorylation of p53 in HeLa cells cotransfected with Plk3-A and p53 expression constructs (data not shown). One explanation for this discrepancy is that the Plk3-A clone is missing a short nucleotide sequence encoding about 30 amino acid residues at the amino terminus when compared with that of murine counterpart (19). Although it is active *in vitro*, Plk3-A may be much less so than the cellular Plk3 toward *in vivo* targets. Thus, it is possible that Plk3-A may not behave as a "wild-type" protein *in vivo*, resulting in only a partial response to the upstream activators. Alternatively, HeLa cells may lack a co-factor(s) that is required for activation of Plk3, or the pathway leading activation of Plk3 is compromised.

It is interesting to note that $Plk3^{K52R}$ also suppressed colony

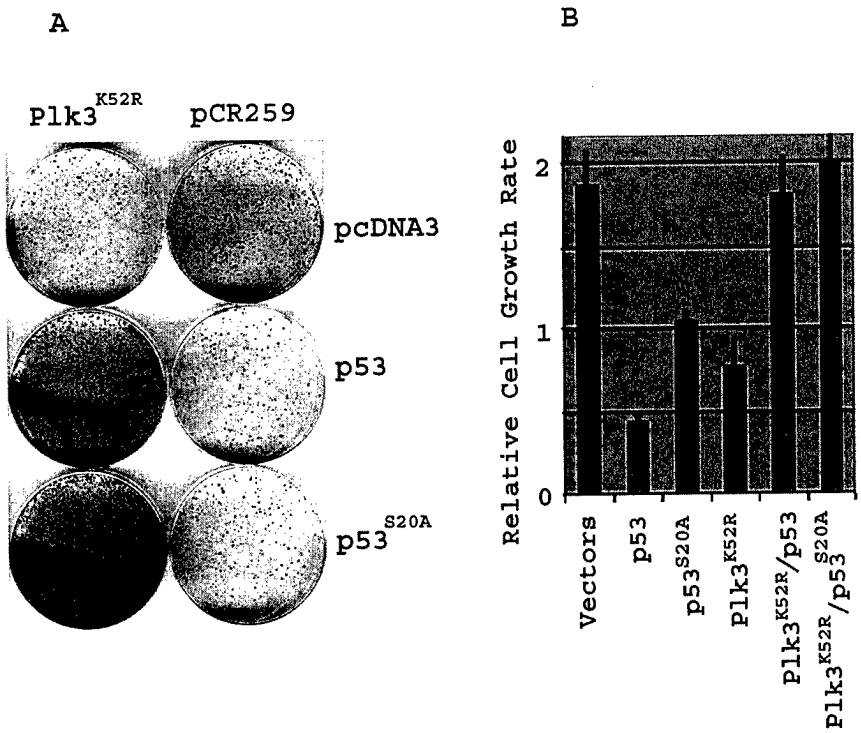


FIG. 7. **Plk3^{K52R} blocks p53-mediated suppression of cell proliferation.** *A*, HeLa cells transfected with p53, p53^{S20A}, and/or Plk3^{K52R} expression constructs were cultured in medium containing G418. Colonies were visualized after staining with crystal violet after 2 weeks' incubation. *B*, HeLa cells transfected with p53, p53^{S20A}, and/or Plk3^{K52R} expression constructs were cultured in the medium containing G418. After 1 week's incubation, cell proliferation rate was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Relative growth rates (A_{570}) were summarized from three independent experiments.

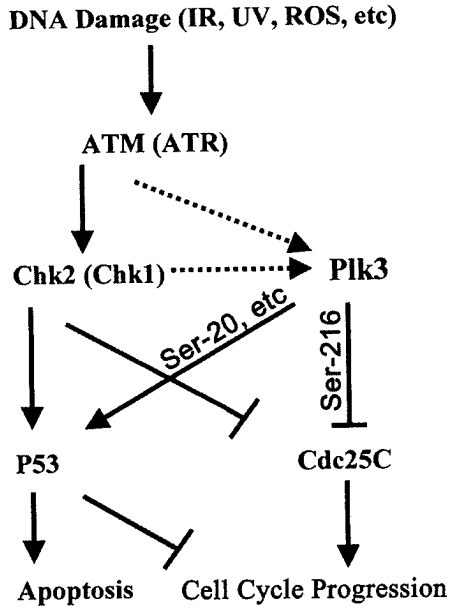


FIG. 8. **A model for the regulation of cell cycle progression by Plk3 in response to DNA damage.** *Dashed arrows* indicate that the direct relationship between these two proteins is not confirmed experimentally.

formation (Fig. 7), which may be partly due to the fact that this mutant protein retains some residual kinase activities toward p53 (Fig. 4*A*) and other *in vitro* substrates such as Cdc25C (2) and α-casein.[2] Given that Plk3-A differs from Plk3^{K52R} by only one amino acid (lysine 52) and that it is more efficient than Plk3^{K52R} in inducing cell cycle arrest and apoptosis (Fig. 1*B*),[2] it is reasonable to conclude that the kinase activity of Plk3 is involved in the regulation of cell proliferation. On the other hand, as shown by the study of Conn *et al.* (25), the COOH-terminal half of Plk3 also plays an important role in induction of apoptosis. In addition, our recent studies indicate that Plk3 is concentrated at the centrosomal region during interphase and that Plk3 appears to be involved in the regulation of microtubule dynamics as well as centrosome function.[3] Therefore, Plk3 may regulate cell proliferation through multiple mechanisms.

Acknowledgments—We thank Chris Herzog for valuable discussions during the course of this work. We also thank Jie Chen for technical assistance.

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Submitted on 8-22-0

Proteasome-dependent down-regulation of p21$^{Waf1/Cip1}$ induced by reactive oxygen species

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Keywords: ROS, p21, proteasome, p53, protein turnover

Running title: Down-regulation of p21 by ROS

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ABSRTACT

Oxidative stress is known to activate p53, resulting in enhanced expression of p53-trageted genes. Here we show that upon hydrogen peroxide (H_2O_2) treatment p21 ($p21^{Waf1/Cip1}$) protein level in GM00637 fibroblast cells was rapidly decreased, reaching the nadir around 3 hours. However, it bounced back within 5 hours to a level higher than that before treatment. Fluorescence microscopic analyses revealed that nuclear p21 was down-regulated during the initial oxidative stress. H_2O_2-induced down-regulation of p21 was p53-independent because p53 was apparently activated within one hour of H_2O_2 treatment. Other genotoxic stresses such as treatment with adriamycin, a DNA damage compound, did not induce a significant decrease in p21 protein level. Pretreatment of GM00637 cells with a proteasome inhibitor lactacystin or MG132 completely blocked H_2O_2-induced p21 down-regulation, suggesting that H_2O_2 treatment accelerated p21 degradation. Conversely, co-treatment of cells with a protein synthesis inhibitor, cycloheximide, and H_2O_2 drastically shortened the half-life of p21. Moreover, H_2O_2 treatment induced a significant increase of G2/M cells, which correlated with the initial down-regulation of p21. Taken together, our studies indicate that oxidative stress induces rapid down-regulation of functional p21 by accelerating its protein turnover.

INTRODUCTION

Reactive oxygen species (ROS) plays an important role in mediating intracellular signaling. Recent studies have demonstrated that intracellular oxygen radicals, generated due to a variety of stimuli, exert profound effects on cell cycle arrest or apoptosis [1;2]. The mechanism by which ROS induces cell cycle arrest or apoptosis remains poorly understood. However, ROS is capable of inducing damage to DNA, which would result in activation of DNA damage checkpoint. The tumor suppressor protein p53, functioning as a genome guardian, is phosphorylated on multiple residues and activated during DNA checkpoint activation induced by genome toxic stresses [3-5]. Increased p53 activity in turn contributes to transactivation of p21, Bax, p53AIP1 and many other genes whose products are responsible for cell growth arrest or programmed cell death [4;6;7]. We have recently demonstrated that H_2O_2 treatment induces phosphorylation of p53 on serines 9, 15, and 20, which is accompanied by increased p53 activity and that ROS-induced phosphorylation of p53 on serine-20 is partly mediated by Plk3 [8]. On the other hand, it has also been shown that p53-dependent apoptosis followed the induction of many genes that are involved in redox regulation [9]. In fact, it has been proposed that p53 may cause cell death by directly stimulating mitochondria to produce an excess amount of toxic ROS in some cells [9]. Therefore, a feedback loop between p53 and ROS may exist, which is presumably to amplify the stress signal, resulting accelerated programmed cell death when damage caused by a genotoxic stress is beyond repair.

The cyclin-dependent kinase (Cdk) inhibitor p21 is a primary target of p53 transcriptional activation. It is an unstable protein, and ubiquitination is thought to be involved in its turnover [10]. It has been shown that polyubiquitinated p21 is degraded rapidly by 26S proteasome [11], which may explain the fact that polyubiquitinated p21 is usually difficult to detect in many cells

[10]. p53 also has a short half-life and is degraded by polyubiquitination (). Interestingly, DNA damage caused by ionizing radiation and ultraviolet light (UV) induces a loss of ubiquitination of p53 but not p21 [10], suggesting that these genotoxic stresses have a differential affect on p53 and p21 protein turnover. However, a recent study indicates that proteasome-mediated turnover of p21 involves no ubiquitination of p21 because a p21 mutant protein lack of a ubiquitination motif remains unstable and increases its abundance upon proteasome inhibition [12].

Here we report that oxidative stress induces a biphasic modulation of p21 protein. H_2O_2 treatment caused a rapid degradation (within one hour) of endogenous p21 that returned to and surpassed the pretreatment level within 5 hours. The ROS-induced initial decrease of p21 was due to an enhanced protein turnover mediated by proteasome because p21 decrease was completely blocked by the pretreatment of cells with either lactacystin or MG132, proteasome-specific inhibitors and because cycloheximide, an inhibitor of new protein synthesis, accelerated the loss of p21 induced by H_2O_2. The biological significance of the biphasic modulation of p21 expression during oxidative stress was discussed.

MATERIALS AND METHODS

Materials. Cell culture medium Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin sulfate) were purchased from Life Technologies Inc. (Rockville, MD). H_2O_2, cyclohexmide, adriamycin, and goat-anti-mouse or goat-anti-rabbit IgGs conjugated with horse-radish peroxidase were purchased from Sigma (St Louis, MO). Lactacystin and MG132 were from Calbiochem (CA). Antibodies to phophorylated p53 and to p21 were purchased from New England Biolab (Boston MA) and

Zymed (Indianapolis, IN), respectively. Antibodies to β-tubulin and to Bax were from Santa Cruz Biotech (Santa Cruz, CA).

Cell culture GM00637 cell line (human fibroblast) was originally from the Coriell Institute for Medical Research. HeLa cells were obtained from American Type Culture Collection (Rockville, MD). Both GM00637 and HeLa Cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 μg/ml penicillin and 50 μg/ml streptomycin sulfate) at 37°C with 5% CO_2. Cells were seeded at 40 to 50% confluence and split at least two times every week.

Treatments. GM00637 or HeLa cells of about 80% confluence were treated with H_2O_2 (200 μM) for 0, 1, 3, 5, and 7 hours (unless otherwise specified). GM00637 cells were also treated with adriamycin (100 μM) for 0, 1, 3, 5 and 7 hours. In some experiments, GM00637 cells were pretreated with protein synthesis inhibitor lactacystin (10 μM) or MG132 (40 μM) or with vehicle dimethyl-sulfoxide (DMSO) for 2 or 4 hours before initiation of H_2O_2 treatment. GM00637 cells were also treated with cycloheximide (100 μg/ml) in the presence or absence of H_2O_2.

Immunoblotting. For Western blotting, cells treated with or without H_2O_2 and/or other chemicals were collected and lysed in a lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 20 mM Na_3VO_4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Equal amounts of cell lysates were analyzed through sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting with first antibodies to human p21, Bax, serine-20 phosphorylated p53, or β-tubulin. After washing, the protein blots were incubated with horseradish peroxidase–conjugated goat secondary antibodies and the specific signals were detected with enhanced chemiluminescence reagents (Amersham

Pharmacia Biotech). Specific signals detected on X-ray films were digitized using a densitometry scanner and analyzed with UN-SCAN-IT$_{TM}$ gel version 5.1 supplied by the provider (Silk Scientific, Orem, Utah).

Flow cytometry. GM00637 cells treated with H_2O_2 for various times were analyzed for cell cycle stages. Briefly, treated cells were collected, fixed with ethanol, and resuspended in phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA). The cells were stained briefly (10 minutes) with DAPI followed by analysis of DNA content. Cells processed for flow cytometry were measured by the ELITE.ESP cytometer/cell sorter (Coulter) as described [13]. Each experiment was repeated at least two times.

Fluorescence microscopy. Localization of p21 was determined by immunofluorescence microscopic analysis. Cells were quickly washed with phosphate-buffered saline and methanol at room temperature before fixing in methanol for 5 minutes. Fixed cells were treated with 0.1% Triton X-100 in PBS for 5 minutes, and then washed three times with ice-cold PBS. After blocking with 2% BSA in PBS for 15 minutes on ice cells were incubated for 1 hour with mouse monoclonal p21 IgGs (5 μg/ml) in 2% BSA solution. Cells were washed three times with PBS and then incubated with Rodamine-conjugated with goat-anti-mouse IgGs at 4°C for 1 hour in the dark. Cells were washed four times with ice-cold PBS and then stained with DAPI (1 μg/ml) for 10 minutes. Fluorescence microscopy was performed on an Olympus AT70 microscope (Spot Diagnostic Instrument) and images were captured using a digital camera using Image System Spot RT software.

RESULTS

We have previously shown that H_2O_2 induces rapid phosphorylation of p53 on multiple sites (serines 9, 15, and 20), and Plk3 appears to mediate at least in part phosphorylation of serine-20 after oxidative stress [8]. The H_2O_2-induced phosphorylation of p53 is correlated with functional activation because p21 expression is induced [8]. However, a detailed time course study showed (Fig. 1A) that p21 protein levels were decreased shortly (1 hour) after H_2O_2 treatment of GM00637 cells. The H_2O_2 induced decrease of p21 levels lasted at least 3 hours post treatment and it returned back to and surpassed the pretreatment levels within 7 hours (Fig. 1A). On the other hand, no significant decrease in the protein level of Bax, also a p53-inducible gene, was detected after H_2O_2 treatment (Fig. 1A). Neither the level of β-tubulin, a house-keeping gene, was significantly changed after the oxidative stress (Fig. 1A). These observations suggest that the effect of H_2O_2 on p21 down-regulation is rather gene-specific.

Next we examined p21 subcellular localization, as well as its level, during oxidative stress using immunefluorescent microscopy. We demonstrated (Fig. 1B) that p21 was primarily localized in the nucleus of untreated cells and that the nuclear p21 was greatly decreased two hours post H_2O_2 treatment. By 7 hours, however, strong p21 staining was again detected in the nucleus, as well as in the cytoplasm of each cell (Fig. 1B), suggesting that functional p21 was modulated during oxidative stress. H_2O_2 treatment apparently caused some shrinkage of cells although they remained attached to the culture plates (Fig. 1B, 7 hours).

It is established that expression of p21 is primarily regulated by p53. One possibility is that the initial decrease of p21 was caused by a drop in p53 activity or its protein level. To test this possibility, we determined the level of p53 phosphorylation on serine-20, which was tightly correlated with its activation [14]. Figure 2 shows that p53 phosphorylation on serine-20 was

induced within 1 hour and remained elevated up to 7 hour post H_2O_2 treatment, indicating that H_2O_2-induced down-regulation of p21 was p53-independent. On the other hand, the subsequent increase in p21 protein level was most likely a result of p53 activation.

To examine whether H_2O_2-induced decrease in p21 protein level was a common phenomenon, we treated HeLa cells with H_2O_2 for various times and cell lysates were analyzed for p21 expression via western blotting. Figure 3A shows that p21 was also down-regulated after H_2O_2 treatment but with a kinetics slightly different from that of GM00637 cells. The p21 protein level was lowest about 1 hour post H_2O_2 treatment. By three hours, p21 started to bounce back, and eventually surpassed the pretreatment level within 7 hours. Reactive oxygen species is known to cause damage to DNA, as well as to proteins. To examine whether double strand DNA break would also have a similar effect on modulation of p21 protein level, GM00637 cells treated with adriamycin for various times were analyzed for p21. Figure 3B shows that adriamycin treatment did not induce a rapid decrease in p21. Neither was p21 protein level accumulated in the cells as rapidly as that observed with H_2O_2 treatment (Fig. 1), suggesting that the decrease in p21 was in response to the stress induced by ROS.

The fast kinetics of p21 down-regulation and its independence of p53 activity suggest the involvement of a post-translational mechanism. We next asked whether proteosome inhibitors could block the decrease of p21 protein level induced by H_2O_2. Figure 4 shows that pretreatment of GM00637 cells with lactacystin for 2 hours completely blocked H_2O_2-induced decrease of p21. Furthermore, pretreatment of the cells with MG132, a different proteasome inhibitor, also prevented p21 from decrease in cells treated with H_2O_2. In fact, p21 protein level was significantly higher in cells pretreated with lactacystin or MG132 (lanes 4-7) than in those pretreated with the vehicle in the absence of H_2O_2 (Fig. 4B, lane 2). Moreover, pretreatment of

cells with proteasome inhibitors for a longer time (4 hours) further stabilized p21 in the presence or absence of H_2O_2 (Fig. 4, lanes 8-11). Quantitative analyses showed that compared with the steady-state level of p21 in untreated cells lactacystin or MG132 stabilized p21 by up to three folds (Fig. 4). Interestingly, neither proteasome inhibitors induced a significant change in β-tubulin level (Fig. 4). Therefore, these observations strongly suggest that rapid p21 decrease during initial phase of oxidative stress was due to enhanced protein degradation.

To further test the notion that protein turnover was responsible for the rapid down-regulation of p21 during oxidative stress, we treated GM00637 cells with cycloheximide, a protein synthesis inhibitor, in the presence or absence of H_2O_2. Figure 5 show that cycloheximide treatment greatly shortened the half-life of p21. In the absence of new protein synthesis the half-life of p21 was about 45 minutes (Figs. 5B and 5C). However, H_2O_2 treatment reduced the half-life of p21 to about 15 minutes (Figs. 5 A and 5C).

To an insight into the biological significance of the initial p21 down-regulation after exposure of cells to ROS we analyzed the cell cycle status via flow cytometry. Figure 6 shows that H_2O_2 treatment induced accumulation of G2/M cells. By 5 hours post treatment almost 30% cells were in G2/M phases of the cell cycle. This G2/M cell increase was preceded with a slightly increase in G1 population and closely correlated with a reduction in S phase cells (Fig. 6).

DISCUSSION

In this report, we have demonstrated that p21 protein level exhibits a biphasic change during oxidative stress. Upon H_2O_2 treatment, p21 undergoes a rapid decrease (for about three hours) before bouncing back to and reaching above the pretreatment level. The initial drop in p21 protein level was not correlated with the p53 activity. In fact, p53 is phosphorylated on

multiple serine residues and activated shortly after H_2O_2 treatment [8]. Proteasome-mediated proteolysis is responsible for p21 down-regulation because proteasome inhibitors completely stabilize p21 after H_2O_2 exposure. Conversely, inhibition of new protein synthesis by cycloheximide accelerates down-regulation of p21. The second phase of p21 modulation is characterized by a steady increase of its protein, reaching a level above the pretreatment within 7 hours. The latter phase correlates very well with the activation of p53. Given that p21 expression is primarily controlled by p53 the elevated p53 activity upon oxidative stress is likely responsible for the second phase of p21 change.

ROS is known to cause damage to proteins by forming oxidized products. Elimination of oxidized proteins is important to the cells because accumulation of damaged proteins would result in cellular dysfunction, disease and aging. Several studies have shown that ubiquitin-proteasome pathway is involved in removal of oxidatively damaged proteins in [15;16]. Thus, H_2O_2-induced p21 degradation may be a consequence of an overall increase in the proteasomal activities that serve to scavenge damaged proteins. However, proteasome-mediated degradation of p21 appears to be rather specific because neither Bax nor p53 nor β-tubulin (an abundant house-keeping gene product) is significantly down-regulated after H_2O_2 treatment (Figs. 1 and 2).

The significance of rapid p21 degradation upon oxidative stress remains unclear. One possibility is that cycling cells may speed up progression of certain phases of the cell cycle when they are under severe oxidative stress. Consistent with other reports [1;2], we demonstrated that ROS induced a G2/M arrest, which correlated with a drop in S phase cells (Fig. 6). The initial p21 degradation during the oxidative stress may play an important role in the accumulation of G2/M cells because it appears to closely follow the down-regulation of p21 (Figs. 1 and 6). p21

is known to inhibit Cdk2 whose activity is essential for S phase progression. The absence of Cdk2 inhibitor would conceivably accelerate S-phase progression. On the other hand, p21 also binds to proliferating cell nuclear antigen (PCNA) that is a processivity factor for DNA polymerases δ and ε and required for DNA synthesis. Rapid down-regulation of p21 would potentially fully activate PCNA and thus accelerate S phase progression, resulting in accumulation of G2/M cells. It has been proposed that inactivation of PCNA by p21 may cause DNA damage during S phase, which could lead to the inhibition of Cdc2 and G2 arrest through a p53-independent mechanism [17].

Recent studies suggest that antioxidants may eliminate cancerous cells or prevent precancerous cells from developing through their ability to induced cell cycle arrest. For example, N-acetylcysteine and vitamin E either inhibit cell proliferation, but not their viability, in a p53-independent manner or induce programmed cells death [18]. Interestingly, absence of p21 increases susceptibility of cells to antioxidant-induced apoptosis [18]. Thus, rapid down-regulation of p21 upon H_2O_2 exposure may serve to prime cells to undergo apoptosis when the damage is beyond repair. It is generally agreed that ROS causes damage to macromolecules including DNA [19]. ROS-induced DNA damage is sufficient to activate cellular sensors or checkpoints, resulting in cell cycle arrest or apoptosis. At present, it remains unclear whether antioxidant- and ROS-induced cell cycle arrest and apoptosis are through different mechanisms or pathways. It is interesting to note that high concentrations of vitamin C, a well-known antioxidant, cause decomposition of lipid hydroperoxides, which can induce highly mutagenic lesions in human DNA [20]. These DNA lesions would inevitably activate DNA damage checkpoint in normal cells, resulting in cell cycle arrest to allow cells to repair these lesions or commit suicide when they are beyond repair.

ACKNOWLEDGEMENT

We thank Jie Chen and Qin Ruan for their technical assistance. We also thank Zbigniew Darzynkiewicz and Frank Traganos for his valuable discussions during the course of this work. This work was supported in part by a Public Health Service Award (RO1-74229) and a Department of Defense award to W.D.

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FIGURE LEGENDS

Figure 1. Biphasic modulation of p21 expression upon treatment with H_2O_2. A, GM00637 cells treated with H_2O_2 for the indicated times were lysed and equal amounts of proteins were blotted for p21. The same blot was stripped and reprobed with antibodies to Bax and β-tubulin. B, GM00637 cells treated with H_2O_2 for the indicated times were fixed and stained with DAPI and an antibody to p21. The p21 signals were detected by indirect immunofluorescent microscopy

Figure 2. Induction of p53 phosphorylation of p53 on serine-20 by H_2O_2. GM00637 cells were treated with H_2O_2 for the indicated times. The treated cells were lysed and equal amounts of proteins were blotted for p53 phosphorylation using an antibody specific to phosphorylated serine-20. The same blot was stripped and reprobed with an antibody to β-tubulin.

Figure 3. Biphasic modulation of p21 by H_2O_2 also occurred in HeLa cells. A, HeLa cells were treated with H_2O_2 for the indicated times. The treated cells were lysed and equal amounts of proteins were blotted for p21 or β-tubulin. B, GM00637 cells were treated with adriamycin for the indicated times. The treated cells were lysed and equal amounts of proteins were blotted for p21 or β-tubulin.

Figure 4. Blocking H_2O_2-induced down-regulation of p21 by proteasome inhibitors. A, GM00637 cells were pretreated with lactacystin or MG132 or a vehicle DMSO for 2 or 4 hours before initiation of H_2O_2 treatment. The cells treated with or without H_2O_2 for 3 hours were lysed and equal amounts of proteins were blotted for p21 or β-tubulin. B, Quantitative analysis of p21 signals as shown in A.

Figure 5. Shortening the half-life of p21 by H_2O_2. GM00637 cells were treated with cycloheximide in the presence (A) or absence (B) of H_2O_2 for the indicated times. The treated cells were lysed and equal amounts of proteins were blotted for p21 or β-tubulin. C, To determine the half-life of p21 in vivo, the signals as shown in A and B were quantified by densitometry.

Figure 6. H_2O_2 treatment induced G2/M arrest. GM00637 cells treated with H_2O_2 for the indicated times were analyzed for their cell cycle status by flow cytometry. Percentage of cells in G1, S, and G2/M was plotted.

ABBREVIATIONS

ROS,	Reactive oxygen species
UV,	Ultraviolet
Cdk,	Cyclin-dependent kinase
DMEM,	Dulbeccos' modified Eagle's medium
FBS,	Fetal bovine serum
SDS-PAGE,	Sodium dodecyle sulfate polyacrylamide gel electrophoresis
PBS,	Phosphate-buffered saline
DMSO,	Dimethylsulfoxide
CAK,	Cdk-activating kinase
DAPI,	4',6-diamidino-2-phenylindole


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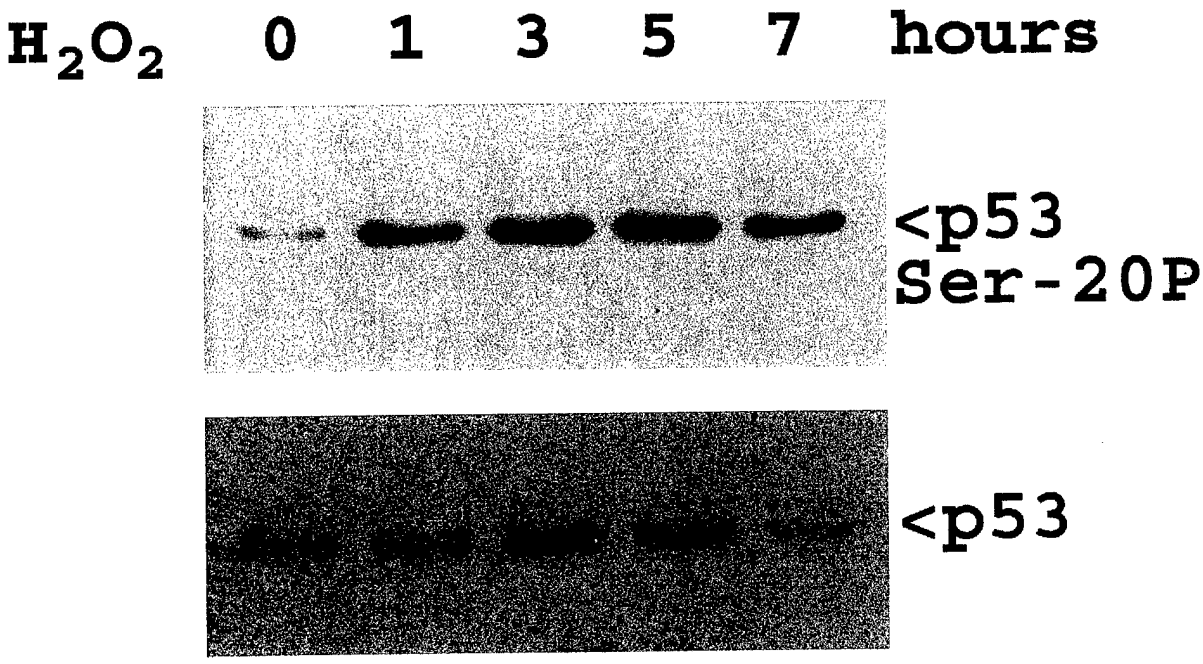
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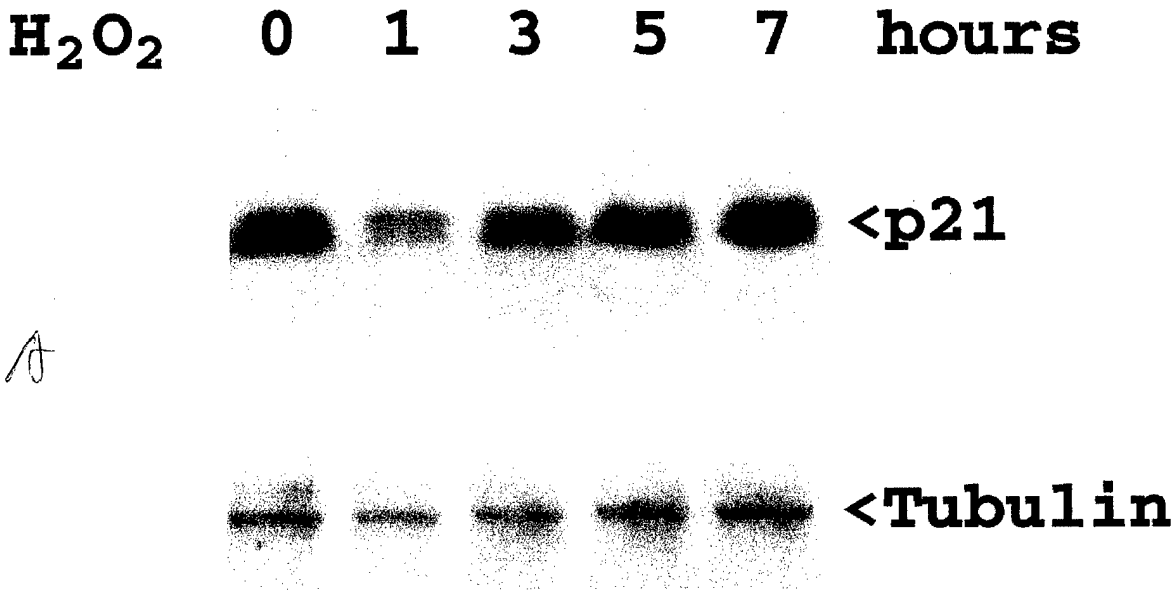

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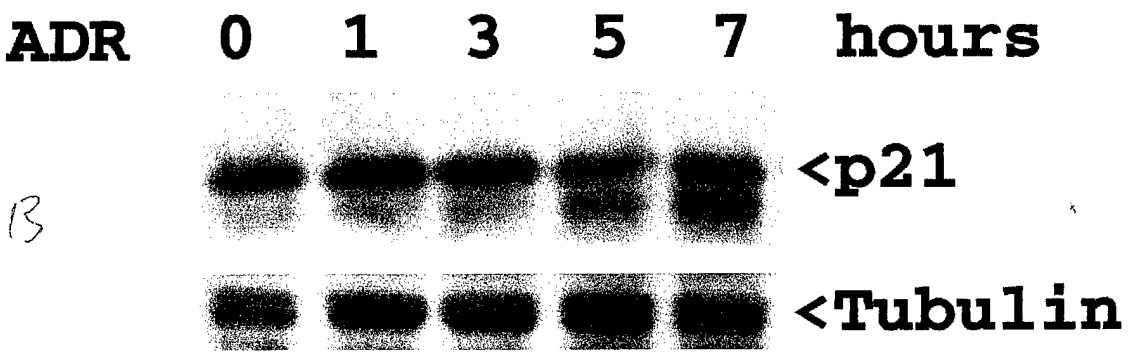
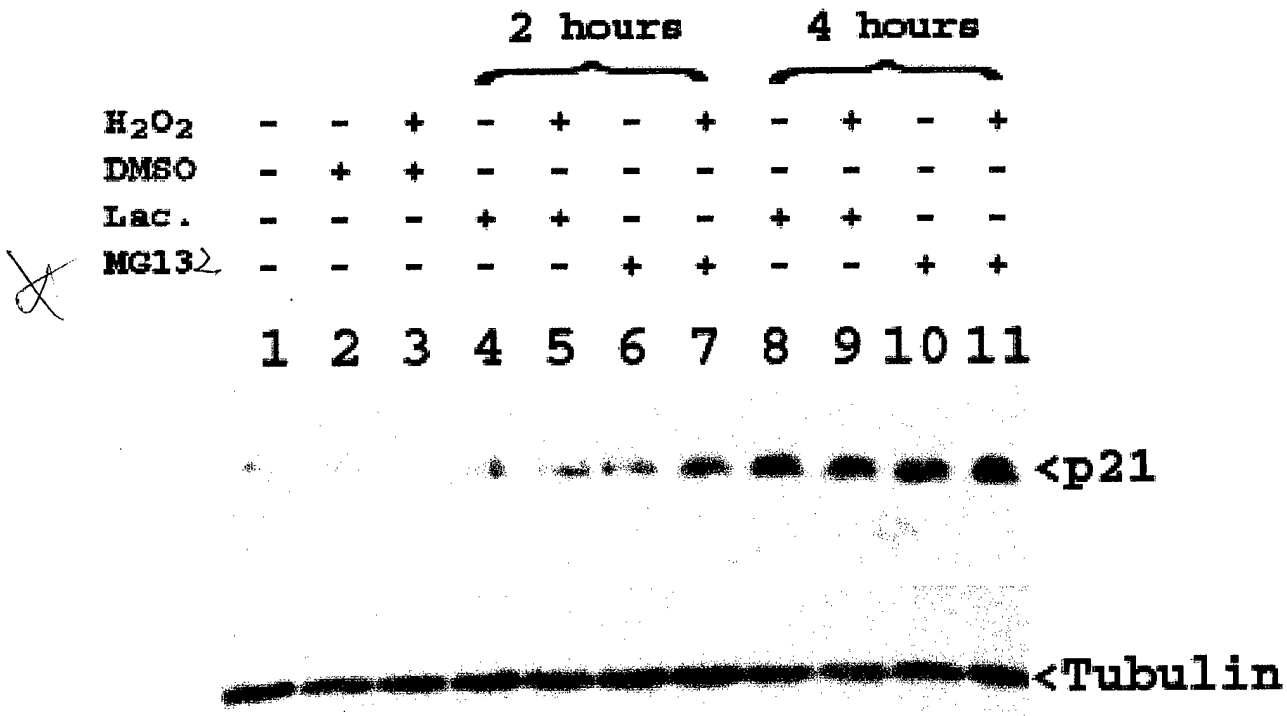
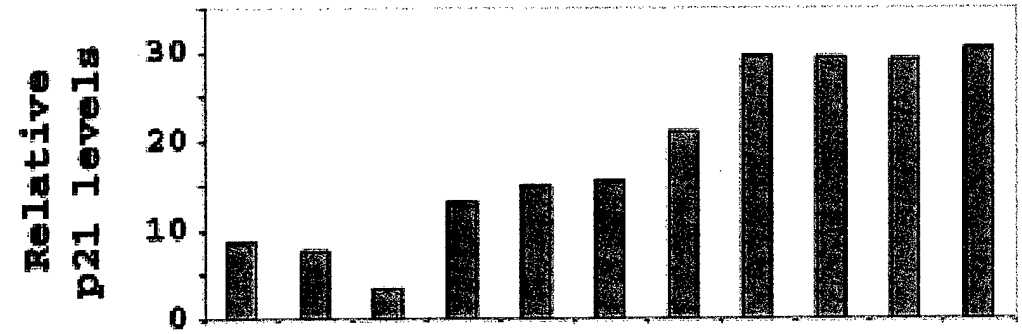


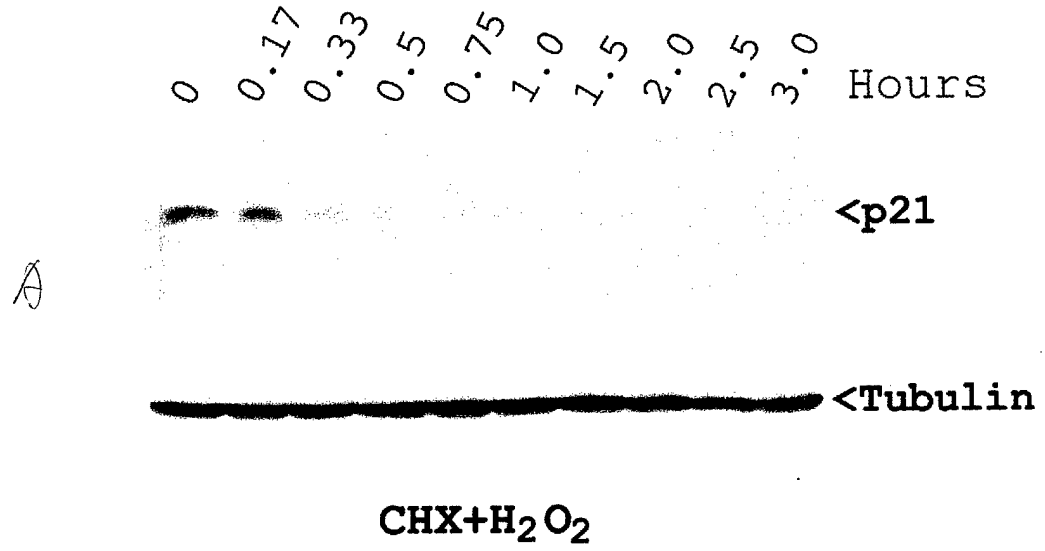
B

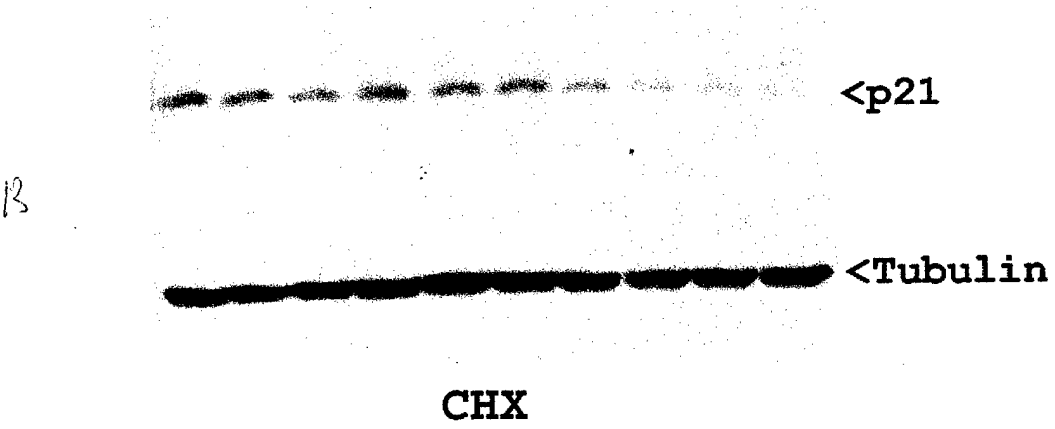

2 hours
4 hours
H2O2 - - + - + - + - + - +
DMSO - + + - - - - - - - -
Lac. - - - + + - - + + - -
MG132 - - - - - + + - - + +
1 2 3 4 5 6 7 8 9 10 11
<p21
<Tubulin

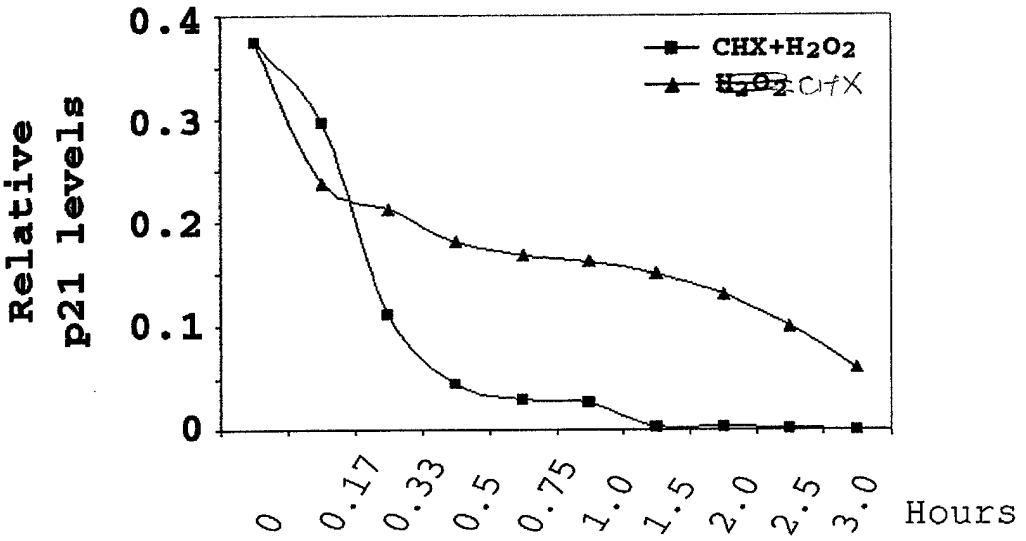
A

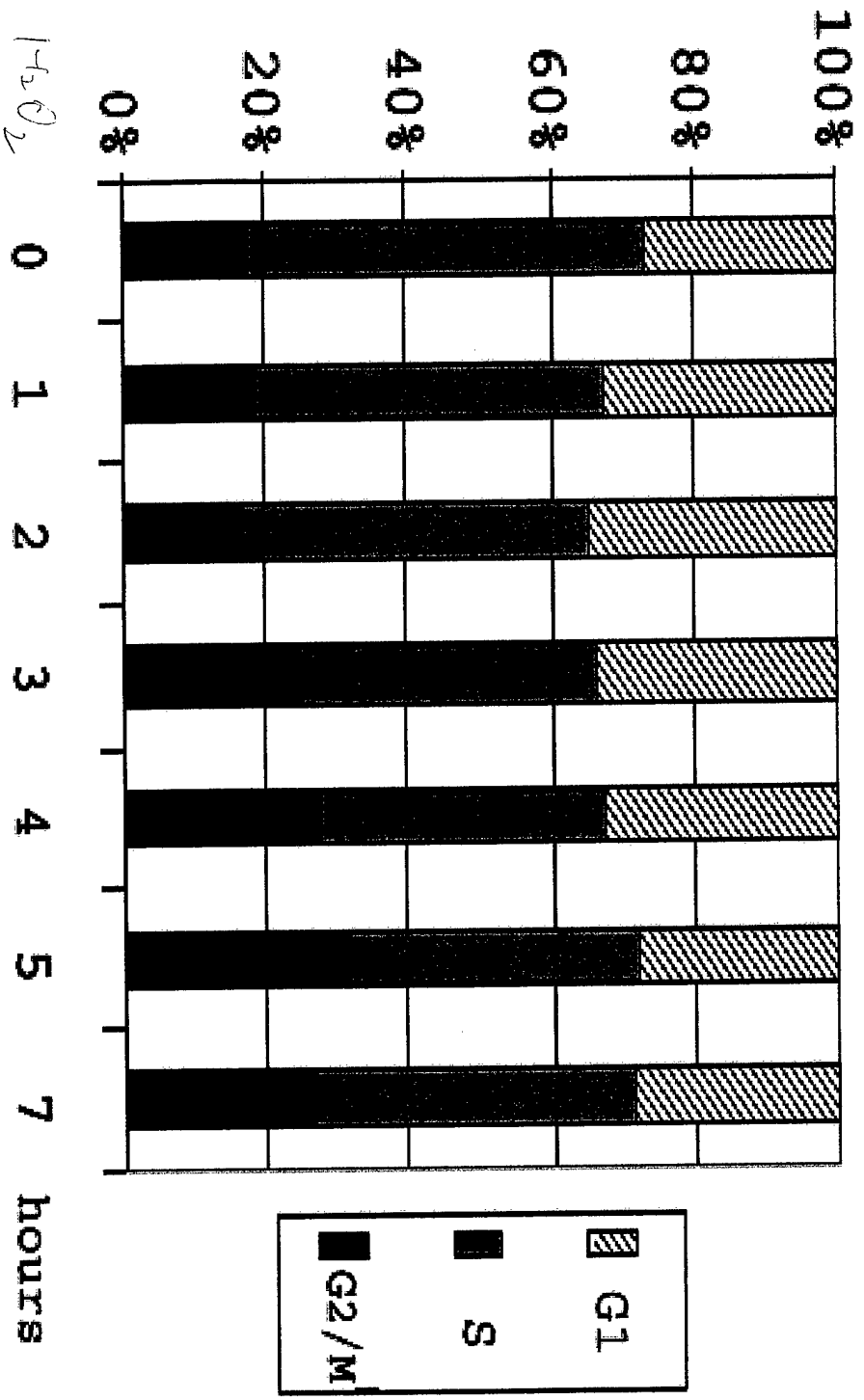

Relative p21 levels
30
20
10
0

B


0 0.17 0.33 0.5 0.75 1.0 1.5 2.0 2.5 3.0
Hours
<p21
<Tubulin
CHX+H2O2


<p21
<Tubulin
CHX


Relative p21 levels
0.4
0.3
0.2
0.1
0
CHX+H2O2
H2O2
0 0.17 0.33 0.5 0.75 1.0 1.5 2.0 2.5 3.0
Hours


Cell Number (%)
0%
20%
40%
60%
80%
100%
0
1
2
3
4
5
7
hours
G2/M
S
G1